ANTISENSE OLIGONUCLEOTIDE MODULATION OF HUMAN PROTEIN KINASE C-δ EXPRESSION

FIELD OF THE INVENTION

This invention relates to compositions and methods for modulating expression of the human protein kinase C-δ gene, a naturally present cellular gene implicated in signal transduction and cellular differentiation. This invention is also directed to methods for modulating differentiation of cells or expression of tumor necrosis factor α; these methods can be used diagnostically or therapeutically. Furthermore, this invention is directed to treatment of conditions or diseases associated with expression of the human protein kinase C-δ gene or tumor necrosis factor α gene.

BACKGROUND OF THE INVENTION

The protein kinase C (PKC) family comprises serine/threonine kinases involved in signal transduction pathways regulating cell proliferation and differentiation. Chronic activation of PKC results in abnormal cellular proliferation and tumor formation. Interest in PKC was stimulated by the finding that PKC is the major, and perhaps only, cellular receptor through which a class of tumor-promoting agents called phorbol esters exert their pleiotropic effects on cells (Gescher *et al.*, *Anti-Cancer Drug Design*, **1989**, *4*, 93-105). Phorbols capable of tumor production can mimic the effect of diacylglycerol (DAG) in activating PKC, suggesting that these tumor promoters act through PKC and that activation of this enzyme is at least partially responsible for the resulting tumorigenesis (Parker *et al.*, *Science*, **1986**, *233*, 853-866).

Increased tumorigenicity is also correlated with overexpression of PKC in cultured cells inoculated into nude mice. A mutant form of PKC induces highly malignant

tumor cells with increased metastatic potential. Sphingosine and related inhibitors of PKC activity have been shown to inhibit tumor cell growth and radiation-induced transformation *in vivo* (Endo *et al.*, *Cancer Research*, **1991**, *51*, 1613-1618); Borek *et al.*, *Proc. Natl. Acad. Sci.*, **1991**, *88*, 1953-1957). A number of experimental or clinically useful anti-cancer drugs show modulatory effects on PKC. Therefore, inhibitors of PKC may be important cancer-preventive or therapeutic agents. PKC has been suggested as a plausible target for more rational design of conventional anti-cancer drugs (Gescher, A. and Dale, I.L., *Anti-Cancer Drug Design*, **1989**, *4*, 93-105).

Experiments also indicate that PKC plays an important role in the pathophysiology of hyperproliferative skin disorders such as psoriasis and skin cancer. Psoriasis is characterized by inflammation, hyperproliferation of the epidermis and decreased differentiation of cells. Various studies indicate a role for PKC in causing these symptoms. PKC stimulation in cultured keratinocytes can be shown to cause hyperproliferation. Inflammation can be induced by phorbol esters and is regulated by PKC. DAG is implicated in the involvement of PKC in dermatological diseases, and is formed to an increased extent in psoriatic lesions.

Inhibitors of PKC have been shown to have both antiproliferative and antiinflammatory effects *in vitro*. Some antipsoriasis drugs, such as cyclosporine A and anthralin, have been shown to inhibit PKC. Inhibition of PKC has been suggested as a therapeutic approach to the treatment of psoriasis (Hegemann, L. and Mahrle, G., *Pharmacology of the Skin*, H. Mukhtar, ed., **1992**, CRC Press, Boca Raton, FL, p. 357-368).

PKC is not a single enzyme, but a family of enzymes. At the present time at least ten isoforms (isozymes) of PKC have been identified: the "conventional" isoforms α, ß, and

γ, the "novel" isoforms δ, ε, η, θ and μ, and the "atypical" isoforms ζ and λ. These isozymes have distinct patterns of tissue and organ localization (see Nishizuka, *FASEB J.*, **1995**, *9*, 484-496 for review) and may serve different physiological functions.

The role of the individual PKC members has been studied by overexpression of the genes and, more recently, using antisense oligonucleotides. Overexpression of PKC-δ has been shown to inhibit cell growth and increased levels are associated with increased tumor potential. For example, PKC-δ is the PKC isoform most represented in murine erythroleukemia (MEL) cells. Incorporation of partially purified PKC-δ protein into permeabilized MEL cells causes a delay in chemically induced differentiation. Thus, it is believed the PKC-δ levels may be important in modulating differentiation in these leukemic cells (Pessino *et al.*, *Biochem J.*, **1995**, *312*, 549-554). However, growth effects may be dependent upon cell type. Modulation of PKC-δ may be particularly useful in hyperproliferative disorders, particularly hematopoietic diseases, such as acute promyelocytic leukemia, and skin disorders, such as psoriasis.

According to the present invention, PKC-δ is also able to modulate tumor necrosis factor α expression. Modulation of PKC-δ may, therefore, also be useful in disease states associated with overexpression of TNF-α, particularly infectious, inflammatory and autoimmune diseases. High levels of plasma TNF-α have been found in infectious diseases such as sepsis syndrome, bacterial meningitis, cerebral malaria, and AIDS; autoimmune diseases such as rheumatoid arthritis, inflammatory bowel disease (including Crohn's disease), sarcoidosis, multiple sclerosis, Kawasaki syndrome, graft-versus-host disease and transplant

(allograft) rejection; organ failure conditions such as adult respiratory distress syndrome, congestive heart failure, acute liver failure and myocardial infarction (Eigler,A., *et al.*, *Immunol. Today*, **1997**, *18*, 487-492). Other diseases in which TNF-α is involved include asthma (Shah,A., *et al.*, *Clinical and Experimental Allergy*, **1995**, *25*, 1038-1044), brain injury following ischemia (Arvin,B., *et al.*, *Ann. NY Acad. Sci.*, **1995**, *765*, 62-71), non-insulin-dependent diabetes mellitus (Hotamisligil,G.S., *et al.*, *Science*, **1993**, *259*, 87-90), insulin-dependent diabetes mellitus (Yang,X.-D., *et al.*, *J. Exp. Med.*, **1994**, *180*, 995-1004), hepatitis (Ksontini,R., *et al.*, *J. Immunol.*, **1998**, *160*, 4082-4089), atopic dermatitis (Sumimoto,S., *et al.*, *Arch. Dis. Child.*, **1992**, *67*, 277-279), and pancreatitis (Norman,J.G., *et al.*, *Surgery*, **1996**, *120*, 515-521). Further, Suganuma,M., *et al.* (*Cancer Res.*, **1996**, *56*, 3711-3715) suggest that inhibitors of TNF-α may be useful for cancer prevention. In addition, elevated TNF-α expression may play a role in obesity (Kern,P.A., *J. Nutr.*, **1997**, *127*, 1917S-1922S). TNF-α was found to be expressed in human adipocytes and increased expression, in general, correlated with obesity.

Two major classes of drugs have been used to induce differentiation. Retinoic acids are used for the treatment of various leukemias (Chomienne, C., *et al.*, *FASEB J.*, **1996**, *10*, 1025-1030) and skin disorders (Orfanos, C.E., *et al.*, *Drugs*, **1997**, *53*, 358-388). A major side effect of retinoic acids is their teratogenicity. Vitamin D3 derivatives are currently being studied for use in skin disorders (Gniadecki, R., *Br. J. Pharmacol.*, **1997**, *120*, 1119-1127; Kobayashi, T., *J. Dermatol. Sci.*, **1998**, 16, 158-164).

Although numerous compounds have been identified as PKC inhibitors (see Hidaka and Hagiwara, *Trends in Pharm. Sci.*, **1987**, *8*, 162-164 for review), few have been found which inhibit PKC specifically, much less specific isozymes of PKC. While the quinoline sulfonamide derivatives such as 1-(5-isoquinolinesulfonyl)-2-methylpiperazine (H-7) inhibit PKC at micromolar concentrations, they exhibit similar enzyme inhibition kinetics for PKC and the CAMP-dependent and cGMP-dependent protein kinases. Staurosporine, an alkaloid product of *Streptomyces* sp., and its analogs, are the most potent *in vitro* inhibitors of PKC identified to date. However, they exhibit only limited selectivity among different protein kinases (Gescher, *Anti-Cancer Drug Design*, **1989**, *4*, 93-105). Certain ceramides and sphingosine derivatives have been shown to have PKC inhibitory activity and to have promise for therapeutic uses, however, there remains a long-felt need for specific inhibitors of the enzymes.

There is also a desire to inhibit specific PKC isozymes, both as a research tool and in diagnosis and treatment of diseases which may be associated with particular isozymes. It is presently believed that different PKC isozymes may be involved in various disease processes depending on the organ or tissue in which they are expressed. Thus far, PKC isozyme specific antisense oligonucleotides have been used to study PKC-α (McGraw, K., *et al.*, *Anti-Cancer Drug Des.*, **1997**, *12*, 315-326), and an antisense oligonucleotide drug, ISIS 3521, targeted to PKC-α is presently in clinical trials and has demonstrated encouraging results in patients with solid tumors. Antisense oligonucleotides have also been used to inhibit PKC-β (Gamard, C.J., *Cell Growth Diff.*, **1994**, *5*, 405-409), PKC-ε (Traub, O., *et al.*, *J. Biol. Chem.*, **1997**, *272*, 31251-31257), and PKC-ζ (Liao, D.F., *J. Biol. Chem.*, **1997**, *272*,

6146-6150). Diaz-Meco Conde *et al.* disclose a peptide corresponding to the pseudo-substrate region of PKC-ζ and oligonucleotides antisense to this isozyme (WO Application 93/20101). Alvaro *et al.* have identified a novel mutant form of PKC associated with tumors and disclose oligonucleotide sequences complementary to the mutant form (WO Application 94/29455).

Specific inhibitors of PKC-δ are believed to be useful for studying the precise role of this isozyme and for therapeutic applications. A compound isolated from *Mallotus philippinensis*, rotterlin, shows some specificity to PKC-δ relative to other PKC family members (Gschwendt, M., *et al.*, *Biochem. Biophys. Res. Commun.*, **1994**, *199*, 93-98). However, this compound also shows some inhibition of other protein kinases including calmodulin-dependent protein kinase III. Antisense oligonucleotides specific for the PKC-δ isozyme have also been used. Liedtke, C.M., et al. (*Am. J. Physiol.*, **1997**, *273*, C1632-C1640) used an oligonucleotide complementary to the translation initiation region of mouse PKC-δ to block α_1-adrenergic activation of Na-K-2Cl cotransport. Pessino, A., *et al.* (*Biochem. J.*, **1995**, *312*, 549-554) used an oligonucleotide complementary to the translation initiation region of PKC-δ to decrease PKC-δ levels and induce differentiation of murine erythroleukemia cells.

There are currently several approaches for directly inhibiting TNF-α expression. These include antibodies, human soluble TNF-α receptor (Camussi,G., *Drugs*, **1998**, *55*, 613-620) and oligonucleotides, including triplex-forming oligonucleotides, ribozymes, and antisense oligonucleotides. Examples of indirect TNF-α inhibitors include phosphodiesterase inhibitors (e.g. pentoxifylline) and metalloprotease inhibitors (Eigler,A., *et al.*, *Immunol.*

Today, **1997**, *18*, 487-492). Indirect inhibitors of TNF-α, such as an inhibitor in the TNF-α signaling pathway, may provide means to inhibit a broad spectrum of activities associated with immune and inflammatory diseases.

There remains a long-felt need for improved compositions and methods for inhibiting PKC-δ gene expression.

SUMMARY OF THE INVENTION

The present invention provides oligonucleotides which are targeted to nucleic acids encoding human PKC-δ and are capable of inhibiting PKC-δ expression. The present invention also provides chimeric oligonucleotides targeted to nucleic acids encoding human PKC-δ. The oligonucleotides of the invention are believed to be useful both diagnostically and therapeutically, and are believed to be particularly useful in the methods of the present invention.

The present invention also comprises methods of inhibiting the expression of human PKC-δ. These methods are believed to be useful both therapeutically and diagnostically as a consequence of the association between PKC-δ inhibition and differentiation and signal transduction leading to TNF-α expression. These methods are also useful as tools, for example, for detecting and determining the role of PKC-δ expression in various cell functions and physiological processes and conditions and for diagnosing conditions associated with PKC-δ or TNF-α expression.

The present invention also comprises methods of modulating differentiation or cell signaling in cells using oligonucleotides of the invention. These methods are believed to be useful, for example, in diagnosing PKC-δ-associated cell differentiation and diseases or conditions

associated with TNF-α expression. Methods of treating abnormal differentiation conditions or inflammatory and immune diseases or conditions are also provided. These methods employ the oligonucleotides of the invention. These methods are believed to be useful both therapeutically and as clinical research and diagnostic tools.

DETAILED DESCRIPTION OF THE INVENTION

Many diseases are associated with unregulated control of cellular differentiation or TNF-α-associated cell signaling. Examples of diseases associated with unregulated control of cellular differentiation include leukemias and skin disorders such as psoriasis. Examples of diseases associated with TNF-α include infectious and inflammatory diseases, particularly diabetes, rheumatoid arthritis, Crohn's disease, pancreatitis, multiple sclerosis, atopic dermatitis and hepatitis. A method of treatment for these diseases could include induction of cellular differentiation or modulation of TNF-α associated cell signaling. The PKC family members are important regulators of cellular proliferation and differentiation. Targeting individual isozymes of PKC could be a useful method to control diseases associated with unregulated cellular differentiation or cell signaling.

Certain abnormal conditions associated with unregulated control of differentiation or cell signaling are believed to be associated with PKC-δ expression and are, therefore believed to be responsive to inhibition of PKC-δ expression. The relationship between an antisense compound such as an oligonucleotide and its complementary nucleic acid target, to which it hybridizes, is commonly referred to as "antisense". "Targeting" an oligonucleotide to a chosen nucleic acid target, in the context of this invention, is a multistep process. The process usually

begins with identifying a nucleic acid sequence whose function is to be modulated. This may be, as examples, a cellular gene (or mRNA made from the gene) whose expression is associated with a particular disease state, or a foreign nucleic acid from an infectious agent. In the present invention, the target is a nucleic acid encoding PKC-δ; in other words, a gene encoding PKC-δ, or mRNA expressed from the PKC-δ gene. mRNA which encodes PKC-δ is presently the preferred target. The targeting process also includes determination of a site or sites within the nucleic acid sequence for the antisense interaction to occur such that modulation of gene expression will result.

In accordance with this invention, persons of ordinary skill in the art will understand that messenger RNA includes not only the information to encode a protein using the three letter genetic code, but also associated ribonucleotides which form a region known to such persons as the 5'-untranslated region, the 3'-untranslated region, the 5' cap region and intron/exon junction ribonucleotides. Thus, oligonucleotides may be formulated in accordance with this invention which are targeted wholly or in part to these associated ribonucleotides as well as to the informational ribonucleotides. The oligonucleotide may therefore be specifically hybridizable with a transcription initiation site region, a translation initiation codon region, a 5' cap region, an intron/exon junction, coding sequences, a translation termination codon region or sequences in the 5'- or 3'-untranslated region. Since, as is known in the art, the translation initiation codon is typically 5'-AUG (in transcribed mRNA molecules; 5'-ATG in the corresponding DNA molecule), the translation initiation codon is also referred to as the "AUG codon," the "start codon" or the "AUG start codon." A minority of genes have a translation initiation codon having the RNA sequence 5'-

GUG, 5'-UUG or 5'-CUG, and 5'-AUA, 5'-ACG and 5'-CUG have been shown to function *in vivo*. Thus, the terms "translation initiation codon" and "start codon" can encompass many codon sequences, even though the initiator amino acid in each instance is typically methionine (in eukaryotes) or formylmethionine (prokaryotes). It is also known in the art that eukaryotic and prokaryotic genes may have two or more alternative start codons, any one of which may be preferentially utilized for translation initiation in a particular cell type or tissue, or under a particular set of conditions. In the context of the invention, "start codon" and "translation initiation codon" refer to the codon or codons that are used *in vivo* to initiate translation of an mRNA molecule transcribed from a gene encoding PKC-δ, regardless of the sequence(s) of such codons. It is also known in the art that a translation termination codon (or "stop codon") of a gene may have one of three sequences, i.e., 5'-UAA, 5'-UAG and 5'-UGA (the corresponding DNA sequences are 5'-TAA, 5'-TAG and 5'-TGA, respectively). The terms "start codon region," "AUG region" and "translation initiation codon region" refer to a portion of such an mRNA or gene that encompasses from about 25 to about 50 contiguous nucleotides in either direction (i.e., 5' or 3') from a translation initiation codon. This region is a preferred target region. Similarly, the terms "stop codon region" and "translation termination codon region" refer to a portion of such an mRNA or gene that encompasses from about 25 to about 50 contiguous nucleotides in either direction (i.e., 5' or 3') from a translation termination codon. This region is a preferred target region. The open reading frame (ORF) or "coding region," which is known in the art to refer to the region between the translation initiation codon and the translation termination codon, is also a region which may be targeted effectively. Other preferred target regions

include the 5' untranslated region (5'UTR), known in the art to refer to the portion of an mRNA in the 5' direction from the translation initiation codon, and thus including nucleotides between the 5' cap site and the translation initiation codon of an mRNA or corresponding nucleotides on the gene and the 3' untranslated region (3'UTR), known in the art to refer to the portion of an mRNA in the 3' direction from the translation termination codon, and thus including nucleotides between the translation termination codon and 3' end of an mRNA or corresponding nucleotides on the gene. The 5' cap of an mRNA comprises an N7-methylated guanosine residue joined to the 5'-most residue of the mRNA via a 5'-5' triphosphate linkage. The 5' cap region of an mRNA is considered to include the 5' cap structure itself as well as the first 50 nucleotides adjacent to the cap. The 5' cap region may also be a preferred target region.

Although some eukaryotic mRNA transcripts are directly translated, many contain one or more regions, known as "introns," which are excised from a pre-mRNA transcript to yield one or more mature mRNA. The remaining (and therefore translated) regions are known as "exons" and are spliced together to form a continuous mRNA sequence. mRNA splice sites, i.e., exon-exon or intron-exon junctions, may also be preferred target regions, and are particularly useful in situations where aberrant splicing is implicated in disease, or where an overproduction of a particular mRNA splice product is implicated in disease. Aberrant fusion junctions due to rearrangements or deletions are also preferred targets. Targeting particular exons in alternatively spliced mRNAs may also be preferred. It has also been found that introns can also be effective, and therefore preferred, target regions for antisense compounds targeted, for example, to DNA or pre-mRNA.

Once the target site or sites have been identified, oligonucleotides are chosen which are sufficiently

complementary to the target, i.e., hybridize sufficiently well and with sufficient specificity, to give the desired modulation.

"Hybridization", in the context of this invention, means hydrogen bonding, also known as Watson-Crick base pairing, between complementary bases, usually on opposite nucleic acid strands or two regions of a nucleic acid strand. Guanine and cytosine are examples of complementary bases which are known to form three hydrogen bonds between them. Adenine and thymine are examples of complementary bases which form two hydrogen bonds between them.

"Specifically hybridizable" and "complementary" are terms which are used to indicate a sufficient degree of complementarity such that stable and specific binding occurs between the DNA or RNA target and the oligonucleotide.

It is understood that an oligonucleotide need not be 100% complementary to its target nucleic acid sequence to be specifically hybridizable. An oligonucleotide is specifically hybridizable when binding of the oligonucleotide to the target interferes with the normal function of the target molecule to cause a loss of utility, and there is a sufficient degree of complementarity to avoid non-specific binding of the oligonucleotide to non-target sequences under conditions in which specific binding is desired, i.e., under physiological conditions in the case of *in vivo* assays or therapeutic treatment and, in the case of *in vitro* assays, under conditions in which the assays are conducted.

Hybridization of antisense oligonucleotides with mRNA interferes with one or more of the normal functions of mRNA. The functions of mRNA to be interfered with include all vital functions such as, for example, translocation of the RNA to the site of protein translation, translation of protein from the RNA, splicing of the RNA to yield one or

more mRNA species, and catalytic activity which may be engaged in by the RNA. Binding of specific protein(s) to the RNA may also be interfered with by antisense oligonucleotide hybridization to the RNA.

The overall effect of interference with mRNA function is modulation of expression of PKC-δ and, in the context of this invention, ultimately modulation of cellular adhesion molecule expression. In the context of this invention "modulation" means either inhibition or stimulation; i.e., either a decrease or increase in expression. This modulation can be measured in ways which are routine in the art, for example by Northern blot assay of mRNA expression, or reverse transcriptase PCR, as taught in the examples of the instant application or by Western blot or ELISA assay of protein expression, or by an immunoprecipitation assay of protein expression. Effects on cell proliferation or tumor cell growth can also be measured, as taught in the examples of the instant application. Inhibition is presently a preferred form of modulation.

The oligonucleotides of this invention can be used in diagnostics, therapeutics, prophylaxis, and as research reagents and in kits. Since the oligonucleotides of this invention hybridize to nucleic acids encoding PKC-δ, sandwich, colorimetric and other assays can easily be constructed to exploit this fact. Provision of means for detecting hybridization of oligonucleotide with the PKC-δ gene or mRNA can routinely be accomplished. Such provision may include enzyme conjugation, radiolabelling or any other suitable detection systems. Kits for detecting the presence or absence of PKC-δ may also be prepared.

The present invention is also suitable for diagnosing abnormal inflammatory states in tissue or other samples from patients suspected of having an inflammatory disease such as rheumatoid arthritis. The ability of the

oligonucleotides of the present invention to inhibit inflammatory processes may be employed to diagnose such states. A number of assays may be formulated employing the present invention, which assays will commonly comprise contacting a tissue sample with an oligonucleotide of the invention under conditions selected to permit detection and, usually, quantitation of such inhibition. In the context of this invention, to "contact" tissues or cells with an oligonucleotide or oligonucleotides means to add the oligonucleotide(s), usually in a liquid carrier, to a cell suspension or tissue sample, either *in vitro* or *ex vivo*, or to administer the oligonucleotide(s) to cells or tissues within an animal.

The oligonucleotides of this invention may also be used for research purposes. For example, the function of a specific gene product in a signaling pathway may be investigated using specific oligonucleotides. Thus, the specific hybridization exhibited by the oligonucleotides may be used for assays, purifications, cellular product preparations and in other methodologies which may be appreciated by persons of ordinary skill in the art.

In the context of this invention, the term "oligonucleotide" refers to an oligomer or polymer of ribonucleic acid or deoxyribonucleic acid. This term includes oligonucleotides composed of naturally-occurring nucleobases, sugars and covalent intersugar (backbone) linkages as well as oligonucleotides having non-naturally-occurring portions which function similarly. Such modified or substituted oligonucleotides are often preferred over native forms because of desirable properties such as, for example, enhanced cellular uptake, enhanced binding to target and increased stability in the presence of nucleases.

The antisense compounds in accordance with this invention preferably comprise from about 5 to about 50

nucleobases. Particularly preferred are antisense oligonucleotides comprising from about 8 to about 30 nucleobases (i.e. from about 8 to about 30 linked nucleosides). As is known in the art, a nucleoside is a base-sugar combination. The base portion of the nucleoside is normally a heterocyclic base. The two most common classes of such heterocyclic bases are the purines and the pyrimidines. Nucleotides are nucleosides that further include a phosphate group covalently linked to the sugar portion of the nucleoside. For those nucleosides that include a pentofuranosyl sugar, the phosphate group can be linked to either the 2', 3' or 5' hydroxyl moiety of the sugar. In forming oligonucleotides, the phosphate groups covalently link adjacent nucleosides to one another to form a linear polymeric compound. In turn the respective ends of this linear polymeric structure can be further joined to form a circular structure, however, open linear structures are generally preferred. Within the oligonucleotide structure, the phosphate groups are commonly referred to as forming the internucleoside backbone of the oligonucleotide. The normal linkage or backbone of RNA and DNA is a 3' to 5' phosphodiester linkage.

Specific examples of preferred antisense compounds useful in this invention include oligonucleotides containing modified backbones or non-natural internucleoside linkages. As defined in this specification, oligonucleotides having modified backbones include those that retain a phosphorus atom in the backbone and those that do not have a phosphorus atom in the backbone. For the purposes of this specification, and as sometimes referenced in the art, modified oligonucleotides that do not have a phosphorus atom in their internucleoside backbone can also be considered to be oligonucleosides.

Preferred modified oligonucleotide backbones include, for example, phosphorothioates, chiral phosphorothioates,

phosphorodithioates, phosphotriesters, aminoalkyl-phosphotriesters, methyl and other alkyl phosphonates including 3'-alkylene phosphonates and chiral phosphonates, phosphinates, phosphoramidates including 3'-amino phosphoramidate and aminoalkylphosphoramidates, thionophosphoramidates, thionoalkylphosphonates, thionoalkylphosphotriesters, and boranophosphates having normal 3'-5' linkages, 2'-5' linked analogs of these, and those having inverted polarity wherein the adjacent pairs of nucleoside units are linked 3'-5' to 5'-3' or 2'-5' to 5'-2'. Various salts, mixed salts and free acid forms are also included.

Representative United States patents that teach the preparation of the above phosphorus-containing linkages include, but are not limited to, U.S. Patent 3,687,808; 4,469,863; 4,476,301; 5,023,243; 5,177,196; 5,188,897; 5,264,423; 5,276,019; 5,278,302; 5,286,717; 5,321,131; 5,399,676; 5,405,939; 5,453,496; 5,455,233; 5,466,677; 5,476,925; 5,519,126; 5,536,821; 5,541,306; 5,550,111; 5,563,253; 5,571,799; 5,587,361; and 5,625,050.

Preferred modified oligonucleotide backbones that do not include a phosphorus atom therein have backbones that are formed by short chain alkyl or cycloalkyl internucleoside linkages, mixed heteroatom and alkyl or cycloalkyl internucleoside linkages, or one or more short chain heteroatomic or heterocyclic internucleoside linkages. These include those having morpholino linkages (formed in part from the sugar portion of a nucleoside); siloxane backbones; sulfide, sulfoxide and sulfone backbones; formacetyl and thioformacetyl backbones; methylene formacetyl and thioformacetyl backbones; alkene containing backbones; sulfamate backbones; methyleneimino and methylenehydrazino backbones; sulfonate and sulfonamide backbones; amide backbones; and others having mixed N, O, S and CH_2 component parts.

Representative United States patents that teach the preparation of the above oligonucleosides include, but are not limited to, U.S. Patent 5,034,506; 5,166,315; 5,185,444; 5,214,134; 5,216,141; 5,235,033; 5,264,562; 5,264,564; 5,405,938; 5,434,257; 5,466,677; 5,470,967; 5,489,677; 5,541,307; 5,561,225; 5,596,086; 5,602,240; 5,610,289; 5,602,240; 5,608,046; 5,610,289; 5,618,704; 5,623,070; 5,663,312; 5,633,360; 5,677,437; and 5,677,439.

In other preferred oligonucleotide mimetics, both the sugar and the internucleoside linkage, i.e., the backbone, of the nucleotide units are replaced with novel groups. The base units are maintained for hybridization with an appropriate nucleic acid target compound. One such oligomeric compound, an oligonucleotide mimetic that has been shown to have excellent hybridization properties, is referred to as a peptide nucleic acid (PNA). In PNA compounds, the sugar-backbone of an oligonucleotide is replaced with an amide containing backbone, in particular an aminoethylglycine backbone. The nucleobases are retained and are bound directly or indirectly to aza nitrogen atoms of the amide portion of the backbone. Representative United States patents that teach the preparation of PNA compounds include, but are not limited to, U.S. Patent 5,539,082; 5,714,331; and 5,719,262. Further teaching of PNA compounds can be found in Nielsen *et al.* (*Science*, **1991**, *254*, 1497-1500).

Most preferred embodiments of the invention are oligonucleotides with phosphorothioate backbones and oligonucleosides with heteroatom backbones, and in particular $-CH_2-NH-O-CH_2-$, $-CH_2-N(CH_3)-O-CH_2-$ [known as a methylene (methylimino) or MMI backbone], $-CH_2-O-N(CH_3)-CH_2-$, $-CH_2-N(CH_3)-N(CH_3)-CH_2-$ and $-O-N(CH_3)-CH_2-CH_2-$ [wherein the native phosphodiester backbone is represented as $-O-P-O-CH_2-$] of the above referenced U.S. Patent 5,489,677, and the amide backbones of the above referenced U.S. Patent

5,602,240. Also preferred are oligonucleotides having morpholino backbone structures of the above-referenced U.S. Patent 5,034,506.

Modified oligonucleotides may also contain one or more substituted sugar moieties. Preferred oligonucleotides comprise one of the following at the 2' position: OH; F; O-, S-, or N-alkyl, O-alkyl-O-alkyl, O-, S-, or N-alkenyl, or O-, S- or N-alkynyl, wherein the alkyl, alkenyl and alkynyl may be substituted or unsubstituted C_1 to C_{10} alkyl or C_2 to C_{10} alkenyl and alkynyl. Particularly preferred are $O[(CH_2)_nO]_mCH_3$, $O(CH_2)_nOCH_3$, $O(CH_2)_2ON(CH_3)_2$, $O(CH_2)_nNH_2$, $O(CH_2)_nCH_3$, $O(CH_2)_nONH_2$, and $O(CH_2)_nON[(CH_2)_nCH_3)]_2$, where n and m are from 1 to about 10. Other preferred oligonucleotides comprise one of the following at the 2' position: C_1 to C_{10} lower alkyl, substituted lower alkyl, alkaryl, aralkyl, O-alkaryl or O-aralkyl, SH, SCH_3, OCN, Cl, Br, CN, CF_3, OCF_3, $SOCH_3$, SO_2CH_3, ONO_2, NO_2, N_3, NH_2, heterocycloalkyl, heterocycloalkaryl, aminoalkylamino, polyalkylamino, substituted silyl, an RNA cleaving group, a reporter group, an intercalator, a group for improving the pharmacokinetic properties of an oligonucleotide, or a group for improving the pharmacodynamic properties of an oligonucleotide, and other substituents having similar properties. A preferred modification includes 2'-methoxyethoxy (2'-O-$CH_2CH_2OCH_3$, also known as 2'-O-(2-methoxyethyl) or 2'-MOE) (Martin *et al.*, *Helv. Chim. Acta* **1995**, *78*, 486-504) i.e., an alkoxyalkoxy group. Further preferred modifications include 2'-dimethylaminooxyethoxy, i.e., a $O(CH_2)_2ON(CH_3)_2$ group, also known as 2'-DMAOE, and 2'-dimethylaminoethoxyethoxy (2'-DMAEOE) as described in examples hereinbelow.

Other preferred modifications include 2'-methoxy (2'-O-CH_3), 2'-aminopropoxy (2'-$OCH_2CH_2CH_2NH_2$) and 2'-fluoro (2'-F). Similar modifications may also be made at other positions on the oligonucleotide, particularly the 3' position of the sugar on the 3' terminal nucleotide or in

2'-5' linked oligonucleotides and the 5' position of 5' terminal nucleotide. Oligonucleotides may also have sugar mimetics such as cyclobutyl moieties in place of the pentofuranosyl sugar. Representative United States patents that teach the preparation of such modified sugars structures include, but are not limited to, U.S. Patent 4,981,957; 5,118,800; 5,319,080; 5,359,044; 5,393,878; 5,446,137; 5,466,786; 5,514,785; 5,519,134; 5,567,811; 5,576,427; 5,591,722; 5,597,909; 5,610,300; 5,627,053l 5,639,873; 5,646,265; 5,658,873; 5,670,633; and 5,700,920.

Oligonucleotides may also include nucleobase (often referred to in the art simply as "base") modifications or substitutions. As used herein, "unmodified" or "natural" nucleobases include the purine bases adenine (A) and guanine (G), and the pyrimidine bases thymine (T), cytosine (C) and uracil (U). Modified nucleobases include other synthetic and natural nucleobases such as 5-methylcytosine (5-me-C or m5c), 5-hydroxymethyl cytosine, xanthine, hypoxanthine, 2-aminoadenine, 6-methyl and other alkyl derivatives of adenine and guanine, 2-propyl and other alkyl derivatives of adenine and guanine, 2-thiouracil, 2-thiothymine and 2-thiocytosine, 5-halouracil and cytosine, 5-propynyl uracil and cytosine, 6-azo uracil, cytosine and thymine, 5-uracil (pseudouracil), 4-thiouracil, 8-halo, 8-amino, 8-thiol, 8-thioalkyl, 8-hydroxyl and other 8-substituted adenines and guanines, 5-halo particularly 5-bromo, 5-trifluoromethyl and other 5-substituted uracils and cytosines, 7-methylguanine and 7-methyladenine, 8-azaguanine and 8-azaadenine, 7-deazaguanine and 7-deazaadenine and 3-deazaguanine and 3-deazaadenine. Further nucleobases include those disclosed in United States Patent No. 3,687,808, those disclosed in the *Concise Encyclopedia Of Polymer Science And Engineering* **1990**, pages 858-859, Kroschwitz, J.I., ed. John Wiley & Sons, those

disclosed by Englisch *et al.* (*Angewandte Chemie, International Edition* **1991**, *30*, 613-722), and those disclosed by Sanghvi, Y.S., Chapter 15, *Antisense Research and Applications* **1993**, pages 289-302, Crooke, S.T. and Lebleu, B., ed., CRC Press. Certain of these nucleobases are particularly useful for increasing the binding affinity of the oligomeric compounds of the invention. These include 5-substituted pyrimidines, 6-azapyrimidines and N-2, N-6 and O-6 substituted purines, including 2-aminopropyladenine, 5-propynyluracil and 5-propynylcytosine. 5-methylcytosine substitutions have been shown to increase nucleic acid duplex stability by 0.6-1.2°C (Sanghvi, Y.S., Crooke, S.T. and Lebleu, B., eds., *Antisense Research and Applications* **1993**, CRC Press, Boca Raton, pages 276-278) and are presently preferred base substitutions, even more particularly when combined with 2'-O-methoxyethyl sugar modifications.

Representative United States patents that teach the preparation of certain of the above noted modified nucleobases as well as other modified nucleobases include, but are not limited to, the above noted U.S. Patent 3,687,808, as well as U.S. Patent 4,845,205; 5,130,302; 5,134,066; 5,175,273; 5,367,066; 5,432,272; 5,457,187; 5,459,255; 5,484,908; 5,502,177; 5,525,711; 5,552,540; 5,587,469; 5,594,121, 5,596,091; 5,614,617; and 5,681,941.

Another modification of the oligonucleotides of the invention involves chemically linking to the oligonucleotide one or more moieties or conjugates which enhance the activity, cellular distribution or cellular uptake of the oligonucleotide. Such moieties include but are not limited to lipid moieties such as a cholesterol moiety (Letsinger *et al.*, *Proc. Natl. Acad. Sci. USA* **1989**, *86*, 6553-6556), cholic acid (Manoharan *et al.*, *Bioorg. Med. Chem. Lett.* **1994**, *4*, 1053-1059), a thioether, e.g., hexyl-

S-tritylthiol (Manoharan *et al.*, *Ann. N.Y. Acad. Sci.* **1992**, *660*, 306-309; Manoharan *et al.*, *Bioorg. Med. Chem. Let.* **1993**, *3*, 2765-2770), a thiocholesterol (Oberhauser *et al.*, *Nucl. Acids Res.* **1992**, *20*, 533-538), an aliphatic chain, e.g., dodecandiol or undecyl residues (Saison-Behmoaras *et al.*, *EMBO J.* **1991**, *10*, 1111-1118; Kabanov *et al.*, *FEBS Lett.* **1990**, *259*, 327-330; Svinarchuk *et al.*, *Biochimie* **1993**, *75*, 49-54), a phospholipid, e.g., di-hexadecyl-*rac*-glycerol or triethylammonium 1,2-di-O-hexadecyl-*rac*-glycero-3-H-phosphonate (Manoharan *et al.*, *Tetrahedron Lett.* **1995**, *36*, 3651-3654; Shea *et al.*, *Nucl. Acids Res.* **1990**, *18*, 3777-3783), a polyamine or a polyethylene glycol chain (Manoharan *et al.*, *Nucleosides & Nucleotides* **1995**, *14*, 969-973), or adamantane acetic acid (Manoharan *et al.*, *Tetrahedron Lett.* **1995**, *36*, 3651-3654), a palmityl moiety (Mishra *et al.*, *Biochim. Biophys. Acta* **1995**, *1264*, 229-237), or an octadecylamine or hexylamino-carbonyl-oxycholesterol moiety (Crooke *et al.*, *J. Pharmacol. Exp. Ther.*, **1996**, *277*, 923-937).

Representative United States patents that teach the preparation of such oligonucleotide conjugates include, but are not limited to, U.S. Patent 4,828,979; 4,948,882; 5,218,105; 5,525,465; 5,541,313; 5,545,730; 5,552,538; 5,578,717, 5,580,731; 5,580,731; 5,591,584; 5,109,124; 5,118,802; 5,138,045; 5,414,077; 5,486,603; 5,512,439; 5,578,718; 5,608,046; 4,587,044; 4,605,735; 4,667,025; 4,762,779; 4,789,737; 4,824,941; 4,835,263; 4,876,335; 4,904,582; 4,958,013; 5,082,830; 5,112,963; 5,214,136; 5,082,830; 5,112,963; 5,214,136; 5,245,022; 5,254,469; 5,258,506; 5,262,536; 5,272,250; 5,292,873; 5,317,098; 5,371,241, 5,391,723; 5,416,203, 5,451,463; 5,510,475; 5,512,667; 5,514,785; 5,565,552; 5,567,810; 5,574,142;

5,585,481; 5,587,371; 5,595,726; 5,597,696; 5,599,923; 5,599,928 and 5,688,941.

The present invention also includes oligonucleotides which are chimeric oligonucleotides. "Chimeric" oligonucleotides or "chimeras," in the context of this invention, are oligonucleotides which contain two or more chemically distinct regions, each made up of at least one nucleotide. These oligonucleotides typically contain at least one region wherein the oligonucleotide is modified so as to confer upon the oligonucleotide increased resistance to nuclease degradation, increased cellular uptake, and/or increased binding affinity for the target nucleic acid. An additional region of the oligonucleotide may serve as a substrate for enzymes capable of cleaving RNA:DNA or RNA:RNA hybrids. By way of example, RNase H is a cellular endonuclease which cleaves the RNA strand of an RNA:DNA duplex. Activation of RNase H, therefore, results in cleavage of the RNA target, thereby greatly enhancing the efficiency of antisense inhibition of gene expression. Cleavage of the RNA target can be routinely detected by gel electrophoresis and, if necessary, associated nucleic acid hybridization techniques known in the art. This RNAse H-mediated cleavage of the RNA target is distinct from the use of ribozymes to cleave nucleic acids. Ribozymes are not comprehended by the present invention.

Examples of chimeric oligonucleotides include but are not limited to "gapmers," in which three distinct regions are present, normally with a central region flanked by two regions which are chemically equivalent to each other but distinct from the gap. A preferred example of a gapmer is an oligonucleotide in which a central portion (the "gap") of the oligonucleotide serves as a substrate for RNase H and is preferably composed of 2'-deoxynucleotides, while the flanking portions (the 5' and 3' "wings") are modified to have greater affinity for the target RNA molecule but

are unable to support nuclease activity (e.g., fluoro- or 2'-O-methoxyethyl- substituted). Chimeric oligonucleotides are not limited to those with modifications on the sugar, but may also include oligonucleosides or oligonucleotides with modified backbones, e.g., with regions of phosphorothioate (P=S) and phosphodiester (P=O) backbone linkages or with regions of MMI and P=S backbone linkages. Other chimeras include "wingmers," also known in the art as "hemimers," that is, oligonucleotides with two distinct regions. In a preferred example of a wingmer, the 5' portion of the oligonucleotide serves as a substrate for RNase H and is preferably composed of 2'-deoxynucleotides, whereas the 3' portion is modified in such a fashion so as to have greater affinity for the target RNA molecule but is unable to support nuclease activity (e.g., 2'-fluoro- or 2'-O-methoxyethyl- substituted), or vice-versa. In one embodiment, the oligonucleotides of the present invention contain a 2'-O-methoxyethyl (2'-O-$CH_2CH_2OCH_3$) modification on the sugar moiety of at least one nucleotide. This modification has been shown to increase both affinity of the oligonucleotide for its target and nuclease resistance of the oligonucleotide. According to the invention, one, a plurality, or all of the nucleotide subunits of the oligonucleotides of the invention may bear a 2'-O-methoxyethyl (-O-$CH_2CH_2OCH_3$) modification. Oligonucleotides comprising a plurality of nucleotide subunits having a 2'-O-methoxyethyl modification can have such a modification on any of the nucleotide subunits within the oligonucleotide, and may be chimeric oligonucleotides. Aside from or in addition to 2'-O-methoxyethyl modifications, oligonucleotides containing other modifications which enhance antisense efficacy, potency or target affinity are also preferred. Chimeric oligonucleotides comprising one or more such modifications are presently preferred.

The oligonucleotides used in accordance with this invention may be conveniently and routinely made through the well-known technique of solid phase synthesis. Equipment for such synthesis is sold by several vendors including Applied Biosystems. Any other means for such synthesis may also be employed; the actual synthesis of the oligonucleotides is well within the talents of the routineer. It is well known to use similar techniques to prepare oligonucleotides such as the phosphorothioates and 2'-alkoxy or 2'-alkoxyalkoxy derivatives, including 2'-O-methoxyethyl oligonucleotides (Martin, P., *Helv. Chim. Acta* **1995**, *78*, 486-504). It is also well known to use similar techniques and commercially available modified amidites and controlled-pore glass (CPG) products such as biotin, fluorescein, acridine or psoralen-modified amidites and/or CPG (available from Glen Research, Sterling, VA) to synthesize fluorescently labeled, biotinylated or other conjugated oligonucleotides.

The antisense compounds of the present invention include bioequivalent compounds, including pharmaceutically acceptable salts and prodrugs. This is intended to encompass any pharmaceutically acceptable salts, esters, or salts of such esters, or any other compound which, upon administration to an animal including a human, is capable of providing (directly or indirectly) the biologically active metabolite or residue thereof. Accordingly, for example, the disclosure is also drawn to pharmaceutically acceptable salts of the nucleic acids of the invention and prodrugs of such nucleic acids. "Pharmaceutically acceptable salts" are physiologically and pharmaceutically acceptable salts of the nucleic acids of the invention: *i.e.*, salts that retain the desired biological activity of the parent compound and do not impart undesired toxicological effects thereto (see, for example, Berge *et*

al., "Pharmaceutical Salts," *J. of Pharma Sci.* **1977**, *66*, 1-19).

For oligonucleotides, examples of pharmaceutically acceptable salts include but are not limited to (a) salts formed with cations such as sodium, potassium, ammonium, magnesium, calcium, polyamines such as spermine and spermidine, *etc.*; (b) acid addition salts formed with inorganic acids, for example hydrochloric acid, hydrobromic acid, sulfuric acid, phosphoric acid, nitric acid and the like; (c) salts formed with organic acids such as, for example, acetic acid, oxalic acid, tartaric acid, succinic acid, maleic acid, fumaric acid, gluconic acid, citric acid, malic acid, ascorbic acid, benzoic acid, tannic acid, palmitic acid, alginic acid, polyglutamic acid, naphthalenesulfonic acid, methanesulfonic acid, p-toluenesulfonic acid, naphthalenedisulfonic acid, polygalacturonic acid, and the like; and (d) salts formed from elemental anions such as chlorine, bromine, and iodine.

The oligonucleotides of the invention may additionally or alternatively be prepared to be delivered in a "prodrug" form. The term "prodrug" indicates a therapeutic agent that is prepared in an inactive form that is converted to an active form (i.e., drug) within the body or cells thereof by the action of endogenous enzymes or other chemicals and/or conditions. In particular, prodrug versions of the oligonucleotides of the invention are prepared as SATE [(S-acetyl-2-thioethyl) phosphate] derivatives according to the methods disclosed in WO 93/24510 to Gosselin *et al.*, published December 9, 1993.

For therapeutic or prophylactic treatment, oligonucleotides are administered in accordance with this invention. Oligonucleotide compounds of the invention may be formulated in a pharmaceutical composition, which may include pharmaceutically acceptable carriers, thickeners,

diluents, buffers, preservatives, surface active agents, neutral or cationic lipids, lipid complexes, liposomes, penetration enhancers, carrier compounds and other pharmaceutically acceptable carriers or excipients and the like in addition to the oligonucleotide. Such compositions and formulations are comprehended by the present invention.

Pharmaceutical compositions comprising the oligonucleotides of the present invention may include penetration enhancers in order to enhance the alimentary delivery of the oligonucleotides. Penetration enhancers may be classified as belonging to one of five broad categories, *i.e.*, fatty acids, bile salts, chelating agents, surfactants and non-surfactants (Lee *et al.*, *Critical Reviews in Therapeutic Drug Carrier Systems* **1991**, *8*, 91-192; Muranishi, *Critical Reviews in Therapeutic Drug Carrier Systems* **1990**, *7*, 1-33). One or more penetration enhancers from one or more of these broad categories may be included.

Various fatty acids and their derivatives which act as penetration enhancers include, for example, oleic acid, lauric acid, capric acid, myristic acid, palmitic acid, stearic acid, linoleic acid, linolenic acid, dicaprate, tricaprate, recinleate, monoolein (a.k.a. 1-monooleoyl-*rac*-glycerol), dilaurin, caprylic acid, arachidonic acid, glyceryl 1-monocaprate, 1-dodecylazacycloheptan-2-one, acylcarnitines, acylcholines, mono- and di-glycerides and physiologically acceptable salts thereof (*i.e.*, oleate, laurate, caprate, myristate, palmitate, stearate, linoleate, *etc.*) (Lee *et al.*, *Critical Reviews in Therapeutic Drug Carrier Systems* **1991**, page 92; Muranishi, *Critical Reviews in Therapeutic Drug Carrier Systems* **1990**, *7*, 1; El-Hariri *et al.*, *J. Pharm. Pharmacol.* **1992** *44*, 651-654).

The physiological roles of bile include the facilitation of dispersion and absorption of lipids and fat-soluble vitamins (Brunton, Chapter 38 *In: Goodman & Gilman's The Pharmacological Basis of Therapeutics*, 9th Ed., Hardman *et al.*, eds., McGraw-Hill, New York, NY, **1996**, pages 934-935). Various natural bile salts, and their synthetic derivatives, act as penetration enhancers. Thus, the term "bile salt" includes any of the naturally occurring components of bile as well as any of their synthetic derivatives.

Complex formulations comprising one or more penetration enhancers may be used. For example, bile salts may be used in combination with fatty acids to make complex formulations.

Chelating agents include, but are not limited to, disodium ethylenediaminetetraacetate (EDTA), citric acid, salicylates (*e.g.*, sodium salicylate, 5-methoxysalicylate and homovanilate), *N*-acyl derivatives of collagen, laureth-9 and *N*-amino acyl derivatives of beta-diketones (enamines) [Lee *et al.*, *Critical Reviews in Therapeutic Drug Carrier Systems* **1991**, page 92; Muranishi, *Critical Reviews in Therapeutic Drug Carrier Systems* **1990**, *7*, 1-33; Buur *et al.*, *J. Control Rel.* **1990**, *14*, 43-51). Chelating agents have the added advantage of also serving as DNase inhibitors.

Surfactants include, for example, sodium lauryl sulfate, polyoxyethylene-9-lauryl ether and polyoxyethylene-20-cetyl ether (Lee *et al.*, *Critical Reviews in Therapeutic Drug Carrier Systems* **1991**, page 92); and perfluorochemical emulsions, such as FC-43 (Takahashi *et al.*, *J. Pharm. Phamacol.* **1988**, *40*, 252-257).

Non-surfactants include, for example, unsaturated cyclic ureas, 1-alkyl- and 1-alkenylazacyclo-alkanone derivatives (Lee *et al.*, *Critical Reviews in Therapeutic*

Drug Carrier Systems **1991**, page 92); and non-steroidal anti-inflammatory agents such as diclofenac sodium, indomethacin and phenylbutazone (Yamashita *et al.*, *J. Pharm. Pharmacol.* **1987**, *39*, 621-626).

As used herein, "carrier compound" refers to a nucleic acid, or analog thereof, which is inert (*i.e.*, does not possess biological activity *per se*) but is recognized as a nucleic acid by *in vivo* processes that reduce the bioavailability of a nucleic acid having biological activity by, for example, degrading the biologically active nucleic acid or promoting its removal from circulation. The coadministration of a nucleic acid and a carrier compound, typically with an excess of the latter substance, can result in a substantial reduction of the amount of nucleic acid recovered in the liver, kidney or other extracirculatory reservoirs, presumably due to competition between the carrier compound and the nucleic acid for a common receptor. In contrast to a carrier compound, a "pharmaceutically acceptable carrier" (excipient) is a pharmaceutically acceptable solvent, suspending agent or any other pharmacologically inert vehicle for delivering one or more nucleic acids to an animal. The pharmaceutically acceptable carrier may be liquid or solid and is selected with the planned manner of administration in mind so as to provide for the desired bulk, consistency, *etc.*, when combined with a nucleic acid and the other components of a given pharmaceutical composition. Typical pharmaceutically acceptable carriers include, but are not limited to, binding agents (*e.g.*, pregelatinized maize starch, polyvinyl-pyrrolidone or hydroxypropyl methylcellulose, *etc.*); fillers (*e.g.*, lactose and other sugars, microcrystalline cellulose, pectin, gelatin, calcium sulfate, ethyl cellulose, polyacrylates or calcium hydrogen phosphate, *etc.*); lubricants (*e.g.*, magnesium

stearate, talc, silica, colloidal silicon dioxide, stearic acid, metallic stearates, hydrogenated vegetable oils, corn starch, polyethylene glycols, sodium benzoate, sodium acetate, *etc.*); disintegrates (*e.g.*, starch, sodium starch glycolate, *etc.*); or wetting agents (*e.g.*, sodium lauryl sulphate, *etc.*). Sustained release oral delivery systems and/or enteric coatings for orally administered dosage forms are described in U.S. Patent 4,704,295; 4,556,552; 4,309,406; and 4,309,404.

The compositions of the present invention may additionally contain other adjunct components conventionally found in pharmaceutical compositions, at their art-established usage levels. Thus, for example, the compositions may contain additional compatible pharmaceutically-active materials such as, *e.g.*, antipruritics, astringents, local anesthetics or anti-inflammatory agents, or may contain additional materials useful in physically formulating various dosage forms of the composition of present invention, such as dyes, flavoring agents, preservatives, antioxidants, opacifiers, thickening agents and stabilizers. However, such materials, when added, should not unduly interfere with the biological activities of the components of the compositions of the invention.

Regardless of the method by which the oligonucleotides of the invention are introduced into a patient, colloidal dispersion systems may be used as delivery vehicles to enhance the *in vivo* stability of the oligonucleotides and/or to target the oligonucleotides to a particular organ, tissue or cell type. Colloidal dispersion systems include, but are not limited to, macromolecule complexes, nanocapsules, microspheres, beads and lipid-based systems including oil-in-water emulsions, micelles, mixed micelles, liposomes and lipid:oligonucleotide complexes of uncharacterized structure. A preferred colloidal

dispersion system is a plurality of liposomes. Liposomes are microscopic spheres having an aqueous core surrounded by one or more outer layers made up of lipids arranged in a bilayer configuration (see, generally, Chonn *et al.*, *Current Op. Biotech.* **1995**, *6*, 698-708).

The pharmaceutical compositions of the present invention may be administered in a number of ways depending upon whether local or systemic treatment is desired and upon the area to be treated. Administration may be topical (including ophthalmic, vaginal, rectal, intranasal, epidermal, and transdermal), oral or parenteral. Parenteral administration includes intravenous drip, subcutaneous, intraperitoneal or intramuscular injection, pulmonary administration, e.g., by inhalation or insufflation, or intracranial, e.g., intrathecal or intraventricular, administration. Oligonucleotides with at least one 2'-O-methoxyethyl modification are believed to be particularly useful for oral administration.

Formulations for topical administration may include transdermal patches, ointments, lotions, creams, gels, drops, suppositories, sprays, liquids and powders. Conventional pharmaceutical carriers, aqueous, powder or oily bases, thickeners and the like may be necessary or desirable. Coated condoms, gloves and the like may also be useful.

Compositions for oral administration include powders or granules, suspensions or solutions in water or non-aqueous media, capsules, sachets or tablets. Thickeners, flavoring agents, diluents, emulsifiers, dispersing aids or binders may be desirable.

Compositions for parenteral administration may include sterile aqueous solutions which may also contain buffers, diluents and other suitable additives. In some cases it may be more effective to treat a patient with an oligonucleotide of the invention in conjunction with other

traditional therapeutic modalities in order to increase the efficacy of a treatment regimen. In the context of the invention, the term "treatment regimen" is meant to encompass therapeutic, palliative and prophylactic modalities. For example, a patient may be treated with conventional chemotherapeutic agents, particularly those used for tumor and cancer treatment. Examples of such chemotherapeutic agents include but are not limited to daunorubicin, daunomycin, dactinomycin, doxorubicin, epirubicin, idarubicin, esorubicin, bleomycin, mafosfamide, ifosfamide, cytosine arabinoside, bis-chloroethylnitrosurea, busulfan, mitomycin C, actinomycin D, mithramycin, prednisone, hydroxyprogesterone, testosterone, tamoxifen, dacarbazine, procarbazine, hexamethylmelamine, pentamethylmelamine, mitoxantrone, amsacrine, chlorambucil, methylcyclohexylnitrosurea, nitrogen mustards, melphalan, cyclophosphamide, 6-mercaptopurine, 6-thioguanine, cytarabine (CA), 5-azacytidine, hydroxyurea, deoxycoformycin, 4-hydroxyperoxycyclophosphoramide, 5-fluorouracil (5-FU), 5-fluorodeoxyuridine (5-FUdR), methotrexate (MTX), colchicine, taxol, vincristine, vinblastine, etoposide, trimetrexate, teniposide, cisplatin and diethylstilbestrol (DES). See, generally, *The Merck Manual of Diagnosis and Therapy*, 15th Ed. **1987**, pp. 1206-1228, Berkow *et al.*, eds., Rahway, N.J. When used with the compounds of the invention, such chemotherapeutic agents may be used individually (*e.g.*, 5-FU and oligonucleotide), sequentially (*e.g.*, 5-FU and oligonucleotide for a period of time followed by MTX and oligonucleotide), or in combination with one or more other such chemotherapeutic agents (*e.g.*, 5-FU, MTX and oligonucleotide, or 5-FU, radiotherapy and oligonucleotide).

The formulation of therapeutic compositions and their subsequent administration is believed to be within the

skill of those in the art. Dosing is dependent on severity and responsiveness of the disease state to be treated, with the course of treatment lasting from several days to several months, or until a cure is effected or a diminution of the disease state is achieved. Optimal dosing schedules can be calculated from measurements of drug accumulation in the body of the patient. Persons of ordinary skill can easily determine optimum dosages, dosing methodologies and repetition rates. Optimum dosages may vary depending on the relative potency of individual oligonucleotides, and can generally be estimated based on EC_{50}s found to be effective *in vitro* and in *in vivo* animal models. In general, dosage is from 0.01 μg to 100 g per kg of body weight, and may be given once or more daily, weekly, monthly or yearly, or even once every 2 to 20 years. Persons of ordinary skill in the art can easily estimate repetition rates for dosing based on measured residence times and concentrations of the drug in bodily fluids or tissues. Following successful treatment, it may be desirable to have the patient undergo maintenance therapy to prevent the recurrence of the disease state, wherein the oligonucleotide is administered in maintenance doses, ranging from 0.01 μg to 100 g per kg of body weight, once or more daily, to once every 20 years.

Thus, in the context of this invention, by "therapeutically effective amount" is meant the amount of the compound which is required to have a therapeutic effect on the treated individual. This amount, which will be apparent to the skilled artisan, will depend upon the age and weight of the individual, the type of disease to be treated, perhaps even the gender of the individual, and other factors which are routinely taken into consideration when designing a drug treatment. A therapeutic effect is assessed in the individual by measuring the effect of the compound on the disease state in the animal. For example,

if the disease to be treated is cancer, therapeutic effects are assessed by measuring the rate of growth or the size of the tumor, or by measuring the production of compounds such as cytokines, production of which is an indication of the progress or regression of the tumor.

The following examples illustrate the present invention and are not intended to limit the same.

EXAMPLES

EXAMPLE 1: Synthesis of Oligonucleotides

Unmodified oligodeoxynucleotides are synthesized on an automated DNA synthesizer (Applied Biosystems model 380B) using standard phosphoramidite chemistry with oxidation by iodine. β-cyanoethyldiisopropyl-phosphoramidites were purchased from Applied Biosystems (Foster City, CA). For phosphorothioate oligonucleotides, the standard oxidation bottle was replaced by a 0.2 M solution of ^{3}H-1,2-benzodithiole-3-one 1,1-dioxide in acetonitrile for the stepwise thiation of the phosphite linkages. The thiation cycle wait step was increased to 68 seconds and was followed by the capping step. Cytosines may be 5-methyl cytosines. (5-methyl deoxycytidine phosphoramidites available from Glen Research, Sterling, VA or Amersham Pharmacia Biotech, Piscataway, NJ)

2'-methoxy oligonucleotides are synthesized using 2'-methoxy β-cyanoethyldiisopropyl-phosphoramidites (Chemgenes, Needham, MA) and the standard cycle for unmodified oligonucleotides, except the wait step after pulse delivery of tetrazole and base is increased to 360 seconds. Other 2'-alkoxy oligonucleotides are synthesized by a modification of this method, using appropriate 2'-modified amidites such as those available from Glen Research, Inc., Sterling, VA.

2'-fluoro oligonucleotides are synthesized as described in Kawasaki *et al.* (*J. Med. Chem.* **1993**, *36*, 831-841). Briefly, the protected nucleoside N^{6}-benzoyl-2'-

deoxy-2'-fluoroadenosine is synthesized utilizing commercially available 9-ß-D-arabinofuranosyladenine as starting material and by modifying literature procedures whereby the 2'-α-fluoro atom is introduced by a S_N2-displacement of a 2'-ß-O-trifyl group. Thus N^6-benzoyl-9-ß-D-arabinofuranosyladenine is selectively protected in moderate yield as the 3',5'-ditetrahydropyranyl (THP) intermediate. Deprotection of the THP and N^6-benzoyl groups is accomplished using standard methodologies and standard methods are used to obtain the 5'-dimethoxytrityl- (DMT) and 5'-DMT-3'-phosphoramidite intermediates.

The synthesis of 2'-deoxy-2'-fluoroguanosine is accomplished using tetraisopropyldisiloxanyl (TPDS) protected 9-ß-D-arabinofuranosylguanine as starting material, and conversion to the intermediate diisobutyryl-arabinofuranosylguanosine. Deprotection of the TPDS group is followed by protection of the hydroxyl group with THP to give diisobutyryl di-THP protected arabinofuranosylguanine. Selective O-deacylation and triflation is followed by treatment of the crude product with fluoride, then deprotection of the THP groups. Standard methodologies are used to obtain the 5'-DMT- and 5'-DMT-3'-phosphoramidites.

Synthesis of 2'-deoxy-2'-fluorouridine is accomplished by the modification of a known procedure in which 2, 2'-anhydro-1-ß-D-arabinofuranosyluracil is treated with 70% hydrogen fluoride-pyridine. Standard procedures are used to obtain the 5'-DMT and 5'-DMT-3'phosphoramidites.

2'-deoxy-2'-fluorocytidine is synthesized via amination of 2'-deoxy-2'-fluorouridine, followed by selective protection to give N^4-benzoyl-2'-deoxy-2'-fluorocytidine. Standard procedures are used to obtain the 5'-DMT and 5'-DMT-3'phosphoramidites.

2'-(2-methoxyethyl)-modified amidites were synthesized according to Martin, P. (*Helv. Chim. Acta* **1995**, *78*, 486-506). For ease of synthesis, the last nucleotide may be a

deoxynucleotide. 2'-O-$CH_2CH_2OCH_3$-cytosines may be 5-methyl cytosines.

Synthesis of 5-Methyl cytosine monomers:

2,2'-Anhydro[1-(β-D-arabinofuranosyl)-5-methyluridine]:

5-Methyluridine (ribosylthymine, commercially available through Yamasa, Choshi, Japan) (72.0 g, 0.279 M), diphenylcarbonate (90.0 g, 0.420 M) and sodium bicarbonate (2.0 g, 0.024 M) were added to DMF (300 mL). The mixture was heated to reflux, with stirring, allowing the evolved carbon dioxide gas to be released in a controlled manner. After 1 hour, the slightly darkened solution was concentrated under reduced pressure. The resulting syrup was poured into diethylether (2.5 L), with stirring. The product formed a gum. The ether was decanted and the residue was dissolved in a minimum amount of methanol (ca. 400 mL). The solution was poured into fresh ether (2.5 L) to yield a stiff gum. The ether was decanted and the gum was dried in a vacuum oven (60°C at 1 mm Hg for 24 h) to give a solid which was crushed to a light tan powder (57 g, 85% crude yield). The material was used as is for further reactions.

2'-O-Methoxyethyl-5-methyluridine:

2,2'-Anhydro-5-methyluridine (195 g, 0.81 M), tris(2-methoxyethyl)borate (231 g, 0.98 M) and 2-methoxyethanol (1.2 L) were added to a 2 L stainless steel pressure vessel and placed in a pre-heated oil bath at 160°C. After heating for 48 hours at 155-160°C, the vessel was opened and the solution evaporated to dryness and triturated with MeOH (200 mL). The residue was suspended in hot acetone (1 L). The insoluble salts were filtered, washed with acetone (150 mL) and the filtrate evaporated. The residue (280 g) was dissolved in CH_3CN (600 mL) and evaporated. A silica gel column (3 kg) was packed in CH_2Cl_2/acetone/MeOH (20:5:3) containing 0.5% Et_3NH. The residue was dissolved in CH_2Cl_2 (250 mL) and adsorbed onto silica (150 g) prior to loading

onto the column. The product was eluted with the packing solvent to give 160 g (63%) of product.

2'-O-Methoxyethyl-5'-O-dimethoxytrityl-5-methyluridine:

2'-O-Methoxyethyl-5-methyluridine (160 g, 0.506 M) was co-evaporated with pyridine (250 mL) and the dried residue dissolved in pyridine (1.3 L). A first aliquot of dimethoxytrityl chloride (94.3 g, 0.278 M) was added and the mixture stirred at room temperature for one hour. A second aliquot of dimethoxytrityl chloride (94.3 g, 0.278 M) was added and the reaction stirred for an additional one hour. Methanol (170 mL) was then added to stop the reaction. HPLC showed the presence of approximately 70% product. The solvent was evaporated and triturated with CH_3CN (200 mL). The residue was dissolved in $CHCl_3$ (1.5 L) and extracted with 2x500 mL of saturated $NaHCO_3$ and 2x500 mL of saturated NaCl. The organic phase was dried over Na_2SO_4, filtered and evaporated. 275 g of residue was obtained. The residue was purified on a 3.5 kg silica gel column, packed and eluted with EtOAc/Hexane/Acetone (5:5:1) containing 0.5% Et_3NH. The pure fractions were evaporated to give 164 g of product. Approximately 20 g additional was obtained from the impure fractions to give a total yield of 183 g (57%).

3'-O-Acetyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methyluridine:

2'-O-Methoxyethyl-5'-O-dimethoxytrityl-5-methyluridine (106 g, 0.167 M), DMF/pyridine (750 mL of a 3:1 mixture prepared from 562 mL of DMF and 188 mL of pyridine) and acetic anhydride (24.38 mL, 0.258 M) were combined and stirred at room temperature for 24 hours. The reaction was monitored by tlc by first quenching the tlc sample with the addition of MeOH. Upon completion of the reaction, as judged by tlc, MeOH (50 mL) was added and the mixture evaporated at 35°C. The residue was dissolved in $CHCl_3$ (800 mL) and extracted with 2x200 mL of saturated sodium bicarbonate and 2x200 mL of saturated NaCl. The water

layers were back extracted with 200 mL of $CHCl_3$. The combined organics were dried with sodium sulfate and evaporated to give 122 g of residue (approx. 90% product). The residue was purified on a 3.5 kg silica gel column and eluted using EtOAc/Hexane(4:1). Pure product fractions were evaporated to yield 96 g (84%).

3'-O-Acetyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methyl-4-triazoleuridine:

A first solution was prepared by dissolving 3'-O-acetyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methyluridine (96 g, 0.144 M) in CH_3CN (700 mL) and set aside. Triethylamine (189 mL, 1.44 M) was added to a solution of triazole (90 g, 1.3 M) in CH_3CN (1 L), cooled to -5°C and stirred for 0.5 h using an overhead stirrer. $POCl_3$ was added dropwise, over a 30 minute period, to the stirred solution maintained at 0-10°C, and the resulting mixture stirred for an additional 2 hours. The first solution was added dropwise, over a 45 minute period, to the later solution. The resulting reaction mixture was stored overnight in a cold room. Salts were filtered from the reaction mixture and the solution was evaporated. The residue was dissolved in EtOAc (1 L) and the insoluble solids were removed by filtration. The filtrate was washed with 1x300 mL of $NaHCO_3$ and 2x300 mL of saturated NaCl, dried over sodium sulfate and evaporated. The residue was triturated with EtOAc to give the title compound.

2'-O-Methoxyethyl-5'-O-dimethoxytrityl-5-methylcytidine:

A solution of 3'-O-acetyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methyl-4-triazoleuridine (103 g, 0.141 M) in dioxane (500 mL) and NH_4OH (30 mL) was stirred at room temperature for 2 hours. The dioxane solution was evaporated and the residue azeotroped with MeOH (2x200 mL). The residue was dissolved in MeOH (300 mL) and transferred to a 2 liter stainless steel pressure vessel. MeOH (400 mL) saturated with NH_3 gas was added and the vessel heated

to 100°C for 2 hours (tlc showed complete conversion). The vessel contents were evaporated to dryness and the residue was dissolved in EtOAc (500 mL) and washed once with saturated NaCl (200 mL). The organics were dried over sodium sulfate and the solvent was evaporated to give 85 g (95%) of the title compound.

N⁴-Benzoyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methyl-cytidine:

2'-O-Methoxyethyl-5'-O-dimethoxytrityl-5-methyl-cytidine (85 g, 0.134 M) was dissolved in DMF (800 mL) and benzoic anhydride (37.2 g, 0.165 M) was added with stirring. After stirring for 3 hours, tlc showed the reaction to be approximately 95% complete. The solvent was evaporated and the residue azeotroped with MeOH (200 mL). The residue was dissolved in $CHCl_3$ (700 mL) and extracted with saturated $NaHCO_3$ (2x300 mL) and saturated NaCl (2x300 mL), dried over $MgSO_4$ and evaporated to give a residue (96 g). The residue was chromatographed on a 1.5 kg silica column using EtOAc/Hexane (1:1) containing 0.5% Et_3NH as the eluting solvent. The pure product fractions were evaporated to give 90 g (90%) of the title compound.

N⁴-Benzoyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methylcytidine-3'-amidite:

N⁴-Benzoyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methylcytidine (74 g, 0.10 M) was dissolved in CH_2Cl_2 (1 L). Tetrazole diisopropylamine (7.1 g) and 2-cyanoethoxy-tetra-(isopropyl)phosphite (40.5 mL, 0.123 M) were added with stirring, under a nitrogen atmosphere. The resulting mixture was stirred for 20 hours at room temperature (tlc showed the reaction to be 95% complete). The reaction mixture was extracted with saturated $NaHCO_3$ (1x300 mL) and saturated NaCl (3x300 mL). The aqueous washes were back-extracted with CH_2Cl_2 (300 mL), and the extracts were combined, dried over $MgSO_4$ and concentrated. The residue obtained was chromatographed on a 1.5 kg silica column

using EtOAc\Hexane (3:1) as the eluting solvent. The pure fractions were combined to give 90.6 g (87%) of the title compound. 5-methyl-2'-deoxycytidine (5-me-C) containing oligonucleotides were synthesized according to published methods (Sanghvi et al., *Nucl. Acids Res.* **1993**, *21*, 3197-3203) using commercially available phosphoramidites (Glen Research, Sterling VA or ChemGenes, Needham MA).

2'-O-(dimethylaminooxyethyl) nucleoside amidites

2'-(Dimethylaminooxyethoxy) nucleoside amidites [also known in the art as 2'-O-(dimethylaminooxyethyl) nucleoside amidites] are prepared as described in the following paragraphs. Adenosine, cytidine and guanosine nucleoside amidites are prepared similarly to the thymidine (5-methyluridine) except the exocyclic amines are protected with a benzoyl moiety in the case of adenosine and cytidine and with isobutyryl in the case of guanosine.

5'-O-tert-Butyldiphenylsilyl-O^2-2'-anhydro-5-methyluridine

O^2-2'-anhydro-5-methyluridine (Pro. Bio. Sint., Varese, Italy, 100.0g, 0.416 mmol), dimethylaminopyridine (0.66g, 0.013eq, 0.0054mmol) were dissolved in dry pyridine (500 ml) at ambient temperature under an argon atmosphere and with mechanical stirring. *tert*-Butyldiphenylchlorosilane (125.8g, 119.0mL, 1.1eq, 0.458mmol) was added in one portion. The reaction was stirred for 16 h at ambient temperature. TLC (Rf 0.22, ethyl acetate) indicated a complete reaction. The solution was concentrated under reduced pressure to a thick oil. This was partitioned between dichloromethane (1 L) and saturated sodium bicarbonate (2x1 L) and brine (1 L). The organic layer was dried over sodium sulfate and concentrated under reduced pressure to a thick oil. The oil was dissolved in a 1:1 mixture of ethyl acetate and ethyl ether (600mL) and the solution was cooled to -10°C. The resulting crystalline

product was collected by filtration, washed with ethyl ether (3x200 mL) and dried (40°C, 1mm Hg, 24 h) to 149g (74.8%) of white solid. TLC and NMR were consistent with pure product.

5'-O-tert-Butyldiphenylsilyl-2'-O-(2-hydroxyethyl)-5-methyluridine

In a 2 L stainless steel, unstirred pressure reactor was added borane in tetrahydrofuran (1.0 M, 2.0 eq, 622 mL). In the fume hood and with manual stirring, ethylene glycol (350 mL, excess) was added cautiously at first until the evolution of hydrogen gas subsided. 5'-O-tert-Butyldiphenylsilyl-O^2-2'-anhydro-5-methyluridine (149 g, 0.311 mol) and sodium bicarbonate (0.074 g, 0.003 eq) were added with manual stirring. The reactor was sealed and heated in an oil bath until an internal temperature of 160 °C was reached and then maintained for 16 h (pressure < 100 psig). The reaction vessel was cooled to ambient and opened. TLC (Rf 0.67 for desired product and Rf 0.82 for ara-T side product, ethyl acetate) indicated about 70% conversion to the product. In order to avoid additional side product formation, the reaction was stopped, concentrated under reduced pressure (10 to 1mm Hg) in a warm water bath (40-100°C) with the more extreme conditions used to remove the ethylene glycol. [Alternatively, once the low boiling solvent is gone, the remaining solution can be partitioned between ethyl acetate and water. The product will be in the organic phase.] The residue was purified by column chromatography (2kg silica gel, ethyl acetate-hexanes gradient 1:1 to 4:1). The appropriate fractions were combined, stripped and dried to product as a white crisp foam (84g, 50%), contaminated starting material (17.4g) and pure reusable starting material 20g. The yield based on starting material less pure recovered starting material was 58%. TLC and NMR were consistent with 99% pure product.

2'-O-([2-phthalimidoxy)ethyl]-5'-*t*-butyldiphenylsilyl-5-methyluridine

5'-O-tert-Butyldiphenylsilyl-2'-O-(2-hydroxyethyl)-5-methyluridine (20g, 36.98mmol) was mixed with triphenylphosphine (11.63g, 44.36mmol) and N-hydroxyphthalimide (7.24g, 44.36mmol). It was then dried over P_2O_5 under high vacuum for two days at 40°C. The reaction mixture was flushed with argon and dry THF (369.8mL, Aldrich, sure seal bottle) was added to get a clear solution. Diethyl-azodicarboxylate (6.98mL, 44.36mmol) was added dropwise to the reaction mixture. The rate of addition is maintained such that resulting deep red coloration is just discharged before adding the next drop. After the addition was complete, the reaction was stirred for 4 hrs. By that time TLC showed the completion of the reaction (ethylacetate:hexane, 60:40). The solvent was evaporated in vacuum. Residue obtained was placed on a flash column and eluted with ethyl acetate:hexane (60:40), to get 2'-O-([2-phthalimidoxy)ethyl]-5'-*t*-butyldiphenylsilyl-5-methyluridine as white foam (21.819, 86%).

5'-O-*tert*-butyldiphenylsilyl-2'-O-[(2-formadoximinooxy)ethyl]-5-methyluridine

2'-O-([2-phthalimidoxy)ethyl]-5'-*t*-butyldiphenylsilyl-5-methyluridine (3.1g, 4.5mmol) was dissolved in dry CH_2Cl_2 (4.5mL) and methylhydrazine (300mL, 4.64mmol) was added dropwise at -10°C to 0°C. After 1 hr the mixture was filtered, the filtrate was washed with ice cold CH_2Cl_2 and the combined organic phase was washed with water, brine and dried over anhydrous Na_2SO_4. The solution was concentrated to get 2'-*O*-(aminooxyethyl) thymidine, which was then dissolved in MeOH (67.5mL). To this formaldehyde (20% aqueous solution, w/w, 1.1eg.) was added and the mixture for 1 hr. Solvent was removed under vacuum; residue

chromatographed to get 5'-O-*tert*-butyldiphenylsilyl-2'-O-[(2-formadoximinooxy) ethyl]-5-methyluridine as white foam (1.95, 78%).

5'-O-*tert*-Butyldiphenylsilyl-2'-O-[N,N-dimethylaminooxyethyl]-5-methyluridine

5'-O-*tert*-butyldiphenylsilyl-2'-O-[(2-formadoximinooxy)ethyl]-5-methyluridine (1.77g, 3.12mmol) was dissolved in a solution of 1M pyridinium p-toluenesulfonate (PPTS) in dry MeOH (30.6mL). Sodium cyanoborohydride (0.39g, 6.13mmol) was added to this solution at 10°C under inert atmosphere. The reaction mixture was stirred for 10 minutes at 10°C. After that the reaction vessel was removed from the ice bath and stirred at room temperature for 2 hr, the reaction monitored by TLC (5% MeOH in CH_2Cl_2). Aqueous $NaHCO_3$ solution (5%, 10mL) was added and extracted with ethyl acetate (2x20mL). Ethyl acetate phase was dried over anhydrous Na_2SO_4, evaporated to dryness. Residue was dissolved in a solution of 1M PPTS in MeOH (30.6mL). Formaldehyde (20% w/w, 30mL, 3.37mmol) was added and the reaction mixture was stirred at room temperature for 10 minutes. Reaction mixture cooled to 10°C in an ice bath, sodium cyanoborohydride (0.39g, 6.13mmol) was added and reaction mixture stirred at 10°C for 10 minutes. After 10 minutes, the reaction mixture was removed from the ice bath and stirred at room temperature for 2 hrs. To the reaction mixture 5% $NaHCO_3$ (25mL) solution was added and extracted with ethyl acetate (2x25mL). Ethyl acetate layer was dried over anhydrous Na_2SO_4 and evaporated to dryness . The residue obtained was purified by flash column chromatography and eluted with 5% MeOH in CH_2Cl_2 to get 5'-O-*tert*-butyldiphenylsilyl-2'-O-[N,N-dimethylaminooxyethyl]-5-methyluridine as a white foam (14.6g, 80%).

2'-O-(dimethylaminooxyethyl)-5-methyluridine

Triethylamine trihydrofluoride (3.91mL, 24.0mmol) was dissolved in dry THF and triethylamine (1.67mL, 12mmol, dry, kept over KOH). This mixture of triethylamine-2HF was then added to 5'-O-*tert*-butyldiphenylsilyl-2'-O-[N,N-dimethylaminooxyethyl]-5-methyluridine (1.40g, 2.4mmol) and stirred at room temperature for 24 hrs. Reaction was monitored by TLC (5% MeOH in CH_2Cl_2). Solvent was removed under vacuum and the residue placed on a flash column and eluted with 10% MeOH in CH_2Cl_2 to get 2'-O-(dimethylaminooxyethyl)-5-methyluridine (766mg, 92.5%).

5'-O-DMT-2'-O-(dimethylaminooxyethyl)-5-methyluridine

2'-O-(dimethylaminooxyethyl)-5-methyluridine (750mg, 2.17mmol) was dried over P_2O_5 under high vacuum overnight at 40°C. It was then co-evaporated with anhydrous pyridine (20mL). The residue obtained was dissolved in pyridine (11mL) under argon atmosphere. 4-dimethylaminopyridine (26.5mg, 2.60mmol), 4,4'-dimethoxytrityl chloride (880mg, 2.60mmol) was added to the mixture and the reaction mixture was stirred at room temperature until all of the starting material disappeared. Pyridine was removed under vacuum and the residue chromatographed and eluted with 10% MeOH in CH_2Cl_2 (containing a few drops of pyridine) to get 5'-O-DMT-2'-O-(dimethylamino-oxyethyl)-5-methyluridine (1.13g, 80%).

5'-O-DMT-2'-O-(2-N,N-dimethylaminooxyethyl)-5-methyluridine-3'-[(2-cyanoethyl)-N,N-diisopropylphosphoramidite]

5'-O-DMT-2'-O-(dimethylaminooxyethyl)-5-methyluridine (1.08g, 1.67mmol) was co-evaporated with toluene (20mL). To the residue N,N-diisopropylamine tetrazonide (0.29g, 1.67mmol) was added and dried over P_2O_5 under high vacuum overnight at 40°C. Then the reaction mixture was dissolved in anhydrous acetonitrile (8.4mL) and 2-cyanoethyl-N,N,N^1,N^1-tetraisopropylphosphoramidite (2.12mL, 6.08mmol)

was added. The reaction mixture was stirred at ambient temperature for 4 hrs under inert atmosphere. The progress of the reaction was monitored by TLC (hexane:ethyl acetate 1:1). The solvent was evaporated, then the residue was dissolved in ethyl acetate (70mL) and washed with 5% aqueous $NaHCO_3$ (40mL). Ethyl acetate layer was dried over anhydrous Na_2SO_4 and concentrated. Residue obtained was chromatographed (ethyl acetate as eluent) to get 5'-O-DMT-2'-O-(2-N,N-dimethylaminooxyethyl)-5-methyluridine-3'-[(2-cyanoethyl)-N,N-diisopropylphosphoramidite] as a foam (1.04g, 74.9%).

2'-dimethylaminoethoxyethoxy (2'-DMAEOE) nucleoside amidites

2'-dimethylaminoethoxyethoxy (2'-DMAEOE) nucleoside amidites (also known in the art as 2'-O-dimethylaminoethoxyethyl) or 2'-DMAEOE nucleoside amidites) are prepared as follows. Other nucleoside amidites are prepared similarly.

2'-*O*-[2(2-N,N-dimethylaminoethoxy)ethyl]-5-methyl uridine

2[2-(Dimethylamino)ethoxy]ethanol (Aldrich, 6.66 g, 50 mmol) is slowly added to a solution of borane in tetra-hydrofuran (1 M, 10 mL, 10 mmol) with stirring in a 100 mL bomb. Hydrogen gas evolves as the solid dissolves. O^2-,2'-anhydro-5-methyluridine (1.2 g, 5 mmol), and sodium bicarbonate (2.5 mg) are added and the bomb is sealed, placed in an oil bath and heated to 155°C for 26 hours. The bomb is cooled to room temperature and opened. The crude solution is concentrated and the residue partitioned between water (200 mL) and hexanes (200 mL). The excess phenol is extracted into the hexane layer. The aqueous layer is extracted with ethyl acetate (3x200 mL) and the combined organic layers are washed once with water, dried over anhydrous sodium sulfate and concentrated. The residue is columned on silica gel using methanol/methylene chloride 1:20 (which has 2% triethylamine) as the eluent.

As the column fractions are concentrated a colorless solid forms which is collected to give the title compound as a white solid.

5'-*O*-dimethoxytrityl-2'-*O*-[2(2-N,N-dimethylaminoethoxy) ethyl)]-5-methyl uridine

To 0.5 g (1.3 mmol) of 2'-*O*-[2(2-N,N-dimethylamino-ethoxy)ethyl)]-5-methyl uridine in anhydrous pyridine (8 mL), triethylamine (0.36 mL) and dimethoxytrityl chloride (DMT-Cl, 0.87 g, 2 eq.) are added and stirred for 1 hour. The reaction mixture is poured into water (200 mL) and extracted with CH_2Cl_2 (2x200 mL). The combined CH_2Cl_2 layers are washed with saturated $NaHCO_3$ solution, followed by saturated NaCl solution and dried over anhydrous sodium sulfate. Evaporation of the solvent followed by silica gel chromatography using MeOH:CH_2Cl_2:Et_3N (20:1, v/v, with 1% triethylamine) gives the title compound.

5'-*O*-Dimethoxytrityl-2'-*O*-[2(2-N,N-dimethylaminoethoxy) ethyl)]-5-methyl uridine-3'-*O*-(cyanoethyl-N,N-diisopropyl) phosphoramidite

Diisopropylaminotetrazolide (0.6 g) and 2-cyanoethoxy-N,N-diisopropyl phosphoramidite (1.1 mL, 2 eq.) are added to a solution of 5'-*O*-dimethoxytrityl-2'-*O*-[2(2-N,N-dimethylaminoethoxy)ethyl)]-5-methyluridine (2.17 g, 3 mmol) dissolved in CH_2Cl_2 (20 mL) under an atmosphere of argon. The reaction mixture is stirred overnight and the solvent evaporated. The resulting residue is purified by silica gel flash column chromatography with ethyl acetate as the eluent to give the title compound.

Oligonucleotides having methylene(methylimino) (MMI) backbones are synthesized according to U.S. Patent 5,378,825, which is coassigned to the assignee of the present invention and is incorporated herein in its entirety. For ease of synthesis, various nucleoside dimers containing MMI linkages were synthesized and incorporated

into oligonucleotides. Other nitrogen-containing backbones are synthesized according to WO 92/20823 which is also coassigned to the assignee of the present invention and incorporated herein in its entirety.

Oligonucleotides having amide backbones are synthesized according to De Mesmaeker *et al.* (*Acc. Chem. Res.* **1995**, *28*, 366-374). The amide moiety is readily accessible by simple and well-known synthetic methods and is compatible with the conditions required for solid phase synthesis of oligonucleotides.

Oligonucleotides with morpholino backbones are synthesized according to U.S. Patent 5,034,506 (Summerton and Weller).

Peptide-nucleic acid (PNA) oligomers are synthesized according to P.E. Nielsen *et al.* (*Science* **1991**, *254*, 1497-1500).

After cleavage from the controlled pore glass column (Applied Biosystems) and deblocking in concentrated ammonium hydroxide at 55°C for 18 hours, the oligonucleotides are purified by precipitation twice out of 0.5 M NaCl with 2.5 volumes ethanol. Synthesized oligonucleotides were analyzed by polyacrylamide gel electrophoresis on denaturing gels and judged to be at least 85% full length material. The relative amounts of phosphorothioate and phosphodiester linkages obtained in synthesis were periodically checked by ^{31}P nuclear magnetic resonance spectroscopy, and for some studies oligonucleotides were purified by HPLC, as described by Chiang *et al.* (*J. Biol. Chem.* **1991**, *266*, 18162). Results obtained with HPLC-purified material were similar to those obtained with non-HPLC purified material.

EXAMPLE 2: Design and Testing of Human PKC-δ Oligonucleotide Sequences for Inhibition of PKC-δ mRNA

Antisense oligonucleotides targeted to human PKC-δ

were designed and synthesized as phosphorothioate oligodeoxynucleotides according to Example 1; oligonucleotide sequences are presented in Table 1. PKC-δ sequence data are from the cDNA sequence published by Aris et al. (*Biochim. Biophys. Acta*, **1993**, *1174*, 171-181); Genbank accession number L07860. This sequence is provided herein as SEQ ID NO: 1.

TABLE 1: Oligonucleotide Sequences Targeted to Human PKC-δ

ISIS NO.	NUCLEOTIDE SEQUENCE[1] (5' -> 3')	SEQ ID NO:	TARGET GENE NUCLEOTIDE CO-ORDINATES[2]	GENE TARGET REGION	% mRNA INHIBITION
10299	GCAGGAACGGCGCCATGGTG	3	0055-0074	AUG	---
10300	CTGGTTCGCCTCGTCCTCGG	4	0114-0133	ORF	28
10301	ATCTGGATGACGCGCCCCTC	5	0251-0270	ORF	30
10302	TTCTTGCAGCGCTCGGCCAG	6	0329-0348	ORF	9
10303	TGCAATCCACGTCCTCCAGG	7	0421-0440	ORF	47
10304	AAGCGGTGCGGCATGTCGAT	8	0480-0499	ORF	13
10305	AAGCGGTGCGGCATGTCGAT	9	0737-0756	ORF	41
10306	GCAGGCTGCCGCAGTGGTCA	10	0790-0809	ORF	13
10307	CCTCCCCAGCAACTCCGGTC	11	1015-1034	ORF	36
10308	AGCGGCCTTTGTCCTGGATG	12	1372-1391	ORF	10
10309	GGCCATCCCGGTCCAACAGC	13	1495-1514	ORF	42
10310	GGTGCTGGCCCGGCTCTCCC	14	1560-1579	ORF	62
10311	GGACCCCGAAAGACCACCAG	15	1648-1667	ORF	72
10312	GTGGCTCCAACCTCCGCTTT	16	1894-1913	ORF	21
10313	AGGAGGTGCTCGAATTTGGG	17	2060-2079	ORF	---

[1] All linkages are phosphorothioate linkages.

[2] Co-ordinates from Genbank Accession No. L07860, locus name "HUMPCKD13X", SEQ ID NO. 1.

A549 cells (obtained from the American Type Culture Collection) were routinely passaged at 80-90% confluency in Dulbecco's modified Eagle's medium (DMEM) containing 1 g glucose/liter and 10% fetal bovine serum (Hyclone, Logan Utah).

A549 cells were treated with phosphorothioate oligonucleotides at 400 nM for four hours in the presence of the cationic lipids DOTMA/DOPE, washed and allowed to recover for an additional 20 hours. Total RNA was extracted and 15 μg of each was resolved on 1% gels and transferred to nylon membranes. The blots were probed with a ^{32}P radiolabeled PKC-δ cDNA probe and then stripped and reprobed with a radiolabeled G3PDH probe to confirm equal RNA loading. The PKC-δ cDNA probe consisted of a 2.1 kb NheI fragment from pBlueBAC-PKC-δ (American Type Culture Collection, Manassas, VA). The glyceraldehyde 3-phosphate dehydrogenase (G3PDH) probe was purchased from Clontech (Palo Alto, CA), Catalog Number 9805-1. PKC-δ transcripts were examined and quantified with a PhosphorImager (Molecular Dynamics, Sunnyvale, CA). Results are shown in Table 1 as percent mRNA inhibition compared to control (untreated). Oligonucleotides 10303 (SEQ ID NO: 7), 10305 (SEQ ID NO: 9), 10309 (SEQ ID NO: 13), 10310 (SEQ ID NO: 14) and 10311 (SEQ ID NO: 15) gave better than 40% reduction of PKC-δ mRNA levels. Oligonucleotides 10310 and 10311 gave better than 60% reduction of PKC-δ.

EXAMPLE 3: Dose Response of Chimeric (deoxy gapped) 2'-O-methoxyethyl PKC-δ Antisense Oligonucleotides on PKC-δ mRNA Levels in NHEK Cells

SEQ ID NO: 15 was synthesized as a uniformly phosphorothioate chimeric oligonucleotide having a centered deoxy gap of eight nucleotides flanked by 2'-O-methoxyethyl (2'-MOE) regions. All 2'-MOE cytosines were 5-me-

cytosines. An additional chimeric oligonucleotide (SEQ ID NO: 18) was synthesized having a randomized sequence and identical base composition for use as a "scrambled" control.

TABLE 2: Nucleotide Sequences of Chemically-modified Human PKC-δ Phosphorothioate Oligonucleotides

ISIS NO.	NUCLEOTIDE SEQUENCE[1] (5' -> 3')	SEQ ID NO:	TARGET GENE NUCLEOTIDE CO-ORDINATES[2]	GENE TARGET REGION
10311	GGACCCCGAAAGACCACCAG	15	1648-1667	ORF
13513	**GGACCCCGAAAGACCACCAG**	15	1648-1667	ORF
17252	**GGACCCCGAAAGACCACCAG**	15	1648-1667	ORF
13514	**AGCCCA**CCGAGACA**CCGAGA**	18	scrambled control	13513

[1] Emboldened residues are 2'-O-methoxyethyl- residues (others are 2'-deoxy-). All 2'-O-methoxyethyl-cytosines are 5-methyl-cytosines; all linkages are phosphorothioate linkages.

[2] Co-ordinates from Genbank Accession No. L07860, locus name "HUMPCKD13X", SEQ ID NO. 1.

NHEK (Normal human epidermal keratinocyte) cells (Clonetics, San Diego CA) were grown in Keratinocyte Growth Medium (KGM) (Gibco BRL, Gaithersburg MD) containing 5 ng/ml of EGF, bovine pituitary extract. NHEK were used at passages 3-5.

NHEK were grown to 60-80% confluency, washed once with basal medium, and then incubated for 4 hours with 5 ml of basal medium containing 10 μg/ml LIPOFECTIN™ (Gibco BRL, Gaithersburg MD) and the indicated concentration of oligonucleotide. mRNA was processed and quantified as described in Example 2.

Oligonucleotides 13513 (SEQ ID NO. 15) and 13514 (SEQ ID NO. 18) were tested at various concentrations. Results are shown in Table 3. Oligonucleotide 13513 (SEQ ID NO. 15) gave approximately 85% inhibition at 300 nM concentration. The IC_{50} is approximately 150 nM. Minimal inhibition was seen with control oligonucleotide 13514 (SEQ ID NO. 18).

TABLE 3: Dose Response of A549 Cells to PKC-δ Antisense Oligonucleotides (ASOs)

ISIS #	SEQ ID NO:	ASO Gene Target	Dose	% mRNA Inhibition
control	---	LIPOFECTIN™ only	---	0%
13513	15	ORF	50 nM	10%
13513	15	"	100 nM	35%
13513	15	"	200 nM	60%
13513	15	"	300 nM	85%
13514	18	scrambled	50 nM	5%
13514	18	"	100 nM	10%
13514	18	"	200 nM	5%
13514	18	"	300 nM	10%

EXAMPLE 4: Time Course of Chimeric Antisense Oligonucleotides on PKC-δ protein levels in NHEK cells

NHEK cells were cultured and treated with oligonucleotide as described in Example 3. Oligonucleotide concentration was 300 nM.

Immunoblot assay:

Cell extracts were electrophoresed on 10% SDS-PAGE mini-gels. The resolved proteins were transferred to Immobilon-P membrane (Millipore, Bedford MA) by electrophoretic transfer and the membrane was blocked for 60 minutes in TBS (Tris-HCl pH 7.4, 150 mM NaCl) containing

5% nonfat milk. The membrane was then incubated for 16 hours at 4°C with monoclonal antibodies raised against PKC-δ (Santa Cruz Biotechnology, Santa Cruz CA) diluted to 0.2 μg/ml in TBS containing 0.2% nonfat milk. This was followed by three washes in TBS plus 0.2% nonfat milk. The membrane was then incubated for one hour with ^{125}I-labelled goat anti-mouse secondary antibody (ICN Radiochemicals, Irvine CA). Membranes were then washed extensively in TBS plus 0.2% nonfat milk. Bands were visualized and quantitated using a Phosphorimager (Molecular Dynamics, Sunnyvale, CA). Results are shown in Table 4. Oligonucleotide 13513 (SEQ ID NO. 15) gave greater 85% inhibition after 120 hours. Minimal inhibition was seen with oligonucleotide 13514 (SEQ ID NO. 18).

TABLE 4: Time Course of Response of Cells to Human PKC-δ Antisense Oligonucleotides (ASOs)

ISIS #	SEQ ID NO:	ASO Gene Target Region	Time	% Protein Inhibition
basal	---	LIPOFECTIN™ only	24 h	0%
basal	---	"	48 h	0%
basal	---	"	72 h	0%
basal	---	"	96 h	0%
basal	---	"	120 h	0%
13513	15	ORF	24 h	20%
13513	15	"	48 h	40%
13513	15	"	72 h	70%
13513	15	"	96 h	80%
13513	15	"	120 h	85%
13514	18	scrambled	24 h	5%
13514	18	"	48 h	---
13514	18	"	72 h	9%

13514	18	"	96 h	10%
13514	18	"	120 h	20%

EXAMPLE 5: Specificity of Chimeric Antisense Oligonucleotides to PKC-δ

The specificity of PKC-δ antisense oligonucleotides was determined by measuring mRNA levels, as described in Example 3, and protein levels, as described in Example 4. mRNA expression levels of PKC-δ, η and ζ were determined 24 hours after treatment. Protein levels of PKC-d, m and ζ were determined 72 hours after oligonucleotide treatment. Only PKC-δ expression was inhibited by oligonucleotide 13513 (SEQ ID NO. 15), demonstrating the specificity of the oligonucleotide.

EXAMPLE 6: Effect of PKC-δ inhibition on TPA responsive genes

Many genes, including late stage markers of differentiation and multiple matrix metalloproteinases, are regulated by TPA. The matrix metalloproteinases (MMPs) are a family of enzymes which have the ability to degrade components of the extracellular matrix (Birkedal-Hansen, *Current Op. Biol.* 7:728 (**1995**)). Many members of the MMP family have been found to have elevated levels of activity in human tumors as well as other disease states (Stetler-Stevenson *et al.*, *Annu. Rev. Cell Biol. 9*:541 (**1993**); Bernhard *et al.*, *Proc. Natl. Acad. Sci. (U.S.A.) 91*:4293 (**1994**)). The following genes were examined for their ability to be induced by the phorbol ester TPA (12-O-tetradecanoylphorbol 13-acetate) and the ability of antisense oligonucleotides targeted to PKC-δ to block this induction: involucrin, keratinocyte transglutaminase, filiggrin, and the matrix metalloproteinases, MMP-1 (interstitial collagenase) and MMP-9 (92 kd gelatinase B).

Only induction of MMP-1 expression was inhibited by PKC-δ antisense oligonucleotides; inhibition was >90%.

EXAMPLE 7: Inhibition of TNF-α expression by antisense inhibition of PKC-δ

NHEK (Normal human epidermal keratinocyte) cells (Clonetics, San Diego CA) were grown in Keratinocyte Growth Medium (KGM) (Gibco BRL, Gaithersburg MD) containing 5 ng/ml of EGF, bovine pituitary extract. NHEK cells were used at passage 4.

NHEK were grown to 60-80% confluency, washed once with basal medium, and then incubated for 4 hours with basal medium containing 6 μg/ml LIPOFECTIN™ (Gibco BRL, Gaithersburg MD) and 200 nM oligonucleotide. Following oligonucleotide treatment, the media was replaced with growth medium and the cells allowed to recover approximately 24 hours. A second oligonucleotide treatment was performed for 5 hours in the presence of LIPOFECTIN™. After 48 hours post-treatment, the medium was removed and the cells were further incubated in Keratinocyte medium containing the supplied growth factors and 100 nM phorbol 12-myristate 13-acetate (PMA, Sigma, St. Louis, MO). mRNA was analyzed 2 hours post-induction with PMA. Protein levels were analyzed approximately 12 hours post-induction.

Total mRNA was isolated using the RNEASY® Mini Kit (Qiagen, Valencia, CA; similar kits from other manufacturers may also be used), separated on a 1% agarose gel, transferred to HYBOND™-N+ membrane (Amersham Pharmacia Biotech, Piscataway, NJ), a positively charged nylon membrane, and probed. A TNF-α probe consisted of the 505 bp EcoRI-HindIII fragment from BBG 18 (R&D Systems, Minneapolis, MN), a plasmid containing human TNF-α cDNA. A glyceraldehyde 3-phosphate dehydrogenase (G3PDH) probe consisted of the 1.06 kb HindIII fragment from pHcGAP

(American Type Culture Collection, Manassas, VA), a plasmid containing human G3PDH cDNA. The restriction fragments were purified from low-melting temperature agarose, as described in Maniatis, T., *et al.*, *Molecular Cloning: A Laboratory Manual*, **1989** and labeled with REDIVUE™ ^{32}P-dCTP (Amersham Pharmacia Biotech, Piscataway, NJ) and PRIME-A-GENE® labelling kit (Promega, Madison, WI). mRNA was quantitated by a PhosphoImager (Molecular Dynamics, Sunnyvale, CA).

Secreted TNF-α protein levels were measured using a human TNF-α ELISA kit (R&D Systems, Minneapolis, MN or Genzyme, Cambridge, MA).

Results are shown in Table 5. ISIS 17252 (SEQ ID NO. 15) was able to reduce TNF-α mRNA expression by approximately 50% and TNF-α protein secretion by approximately 90%. Antisense oligonucleotides to other PKC isoforms including α, ε, and ξ did not reduce TNF-α mRNA or protein levels.

TABLE 5: Inhibition of TNF-α mRNA expression and TNF-α protein secretion by a PKC-δ antisense oligonucleotide

ISIS No:	SEQ ID NO:	GENE TARGET REGION	% mRNA EXPRESSION	% PROTEIN SECRETION
basal	---	---	1%	0%
induced	---	---	100%	100%
17252	15	ORF	54%	12%

EXAMPLE 8: Effect of Antisense Inhibitors of TNF-α in a Murine Model for Non-Insulin-dependent Diabetes Mellitus

The db/db mouse model, a standard model for non-insulin-dependent diabetes mellitus (NIDDM; Hotamisligil,G.S., *et al.*, *Science*, **1993**, *259*, 87-90), was used to assess the activity of TNF-α antisense

oligonucleotides on blood glucose levels and TNF-α mRNA levels in whole mice. These mice have elevated blood glucose levels and TNF-α mRNA levels compared to wild type mice. Female db/db mice and wild-type littermates were purchased from Jackson Laboratories (Bar Harbor, ME). The effect on oligonucleotide 15931 (SEQ ID NO. 19) on blood glucose levels was determined. For determination of TNF-α mRNA levels, oligonucleotide 15931 (SEQ ID NO. 19), a uniformly modified phosphorothioate oligodeoxynucleotide, was compared to oligonucleotide 25302 (SEQ ID NO. 19), a mixed phosphorothioate/phosphodiester chimeric oligonucleotide having regions of 2'-O-methoxyethyl (2'-MOE) nucleotides and deoxynucleotides. The sequences and chemistries are shown in Table 6. Oligonucleotide 18154 (SEQ ID NO. 20) is an antisense mixed phosphorothioate/phosphodiester chimeric oligonucleotide, having regions of 2'-O-methoxyethyl (2'-MOE) nucleotides and deoxynucleotides, targeted to the human vascular cell adhesion molecule-1 (VCAM-1) and was used as an unrelated target control.

TABLE 6: Nucleotide Sequence of TNF-α Antisense Oligonucleotides

ISIS NO.	NUCLEOTIDE SEQUENCE[1] (5' -> 3')	SEQ ID NO:	TARGET GENE NUCLEOTIDE CO-ORDINATES[2]	GENE TARGET REGION
15931	AACCCATCGGCTGGCACCAC	19	5891-5910	coding
25302	**AACCC**ATCGGCTGGC**ACCAC**	19	5891-5910	coding
18154	**TCAAG**CAGTGCCACC**GATCC**	20	target control	

[1] Emboldened residues are 2'-methoxyethyl residues. All 2'-methoxyethyl cytosines and 2'-deoxy cytosines residues are 5-methyl-cytosines; all linkages are phosphorothioate linkages.

[2] Co-ordinates from Genbank Accession No. Y00467, locus name "MMTNFAB".

db/db mice, six to ten weeks old, were dosed intraperitoneally with oligonucleotide every other day for 2 weeks at 10 mg/kg. The mice were fasted for seven hours prior to administration of the oligonucleotide. The mice were bled via retro orbital sinus every other day, and glucose measurements were performed on the blood. Results are shown in Table 7. Oligonucleotide 15931 (SEQ ID NO. 19) was able to reduce blood glucose levels in db/db mice to levels comparable with wild type mice. Food intake between wild type mice, treated and untreated, did not differ. Food intake between db/db mice, treated and untreated, although higher than wild type mice, did not differ significantly.

Samples of the fat (adipose) tissue from the inguinal fat pads were taken for RNA extraction. RNA was extracted according to *Current Protocols in Molecular Biology*, **1997**, Ausubel, F., *et al.* ed., John Wiley & Sons. RNA was purified using the RNA clean up procedure of the RNEASY® Mini kit (Qiagen, Valencia, CA). TNF-α mRNA levels were measured using the RIBOQUANT® kit (PharMingen, San Diego, CA) with 15 μg of RNA per lane. The probe used was from the mCK-3b Multi-Probe Template set (PharMingen, San Diego, CA) labelled with [α^{32}P]UTP (Amersham Pharmacia Biotech, Piscataway, NJ). Results are shown in Table 8. Both oligonucleotide 15931 (SEQ ID NO. 19) and 25302 (SEQ ID NO. 19) were able to reduce TNF-α levels in fat, with 25302 (SEQ ID NO. 19) reducing TNF-α to nearly wild-type levels.

Inhibition of TNF-α has been shown to be effective in a non-insulin diabetes mellitus model.

TABLE 7: Level of Blood Glucose in Normal and db/db Mice After Treatment with TNF-α Antisense Oligonucleotides

Mouse Strain	ISIS #	SEQ ID NO:	ASO Gene Target	Time (days)	blood glucose (mg/dL)
wild type	---	---	---	1	140
"	15931	19	coding	"	138
db/db	---	---	---	1	260
"	15931	19	coding	"	254
wild type	---	---	---	9	175
"	15931	19	coding	"	163
db/db	---	---	---	9	252
"	15931	19	coding	"	128

TABLE 8: Level of TNF-α mRNA in Fat of db/db Mice After Treatment with TNF-α Antisense Oligonucleotides

ISIS No:	SEQ ID NO:	GENE TARGET REGION	% mRNA EXPRESSION
wt saline	---	---	100%
db/db saline	---	---	362%
18154	20	control	130%
15931	19	coding	210%
25302	19	coding	417%

EXAMPLE 9: Effect of Antisense Inhibition of TNF-α in a Murine Model for Rheumatoid Arthritis

Collagen-induced arthritis (CIA) was used as a murine model for arthritis (Mussener,A., *et al., Clin. Exp. Immunol.*, **1997**, *107*, 485-493). Female DBA/1LacJ mice (Jackson Laboratories, Bar Harbor, ME) between the ages of 6 and 8 weeks were used to assess the activity of TNF-α antisense oligonucleotides.

On day 0, the mice were immunized at the base of the tail with 100 μg of bovine type II collagen which is emulsified in Complete Freund's Adjuvant (CFA). On day 7, a second booster dose of collagen was administered by the same route. On day 14, the mice were injected subcutaneously with 100 μg of LPS. Oligonucleotide was administered intraperitoneally daily (10 mg/kg bolus) starting on day -3 (three days before day 0) and continuing for the duration of the study.

Weights were recorded weekly. Mice were inspected daily for the onset of CIA. Paw widths are rear ankle widths of affected and unaffected joints were measured three times a week using a constant tension caliper. Limbs were clinically evaluated and graded on a scale from 0-4 (with 4 being the highest).

Oligonucleotide 25302 (SEQ ID NO. 19) was compared to a saline control. The antisense TNF-α oligonucleotide reduced the incidence of CIA from 70% for the saline control to 40% for the oligonucleotide. The severity of the disease (based on the mean score of the limbs) was also reduced from 3.2 for the saline control to 2.1 for the oligonucleotide.

Inhibition of TNF-α has been shown to be effective in a rheumatoid arthritis model.

EXAMPLE 10: Effect of Antisense Inhibition of TNF-α in a Murine Model for Contact Sensitivity

Contact sensitivity is a type of immune response resulting from contact of the surface of the skin with a sensitizing chemical. A murine model for contact sensitivity is widely used to develop therapies for chronic inflammation, autoimmune disorder, and organ transplant rejection (Goebeler,M., *et al.*, *Int Arch. Allergy Appl. Immunol.*, **1990**, *93*, 294-299). One example of such a disease is atopic dermatitis. Female Balb/c mice between

the ages of 8 and 12 weeks are used to assess the activity of TNF-α antisense oligonucleotides in a contact sensitivity model.

Balb/c mice receive injections of oligonucleotide drug in saline via i.v. injection into the tail vein. The abdomen of the mice is shaved using an Oster hair clipper. The animals are anesthesized using isoflurane, and 25 µl of 0.2% 2,4-dinitrofluorobenzene (DNFB) in 4:1 acetone:olive oil is applied to the shaved abdomen two days in a row. After five days, 10 ml of 0.2% DNFB in the same vehicle is applied to the right ear. After each exposure, the mouse is suspended in air for two minutes to allow the DNFB to absorb into the skin. 24 and 48 hours after application of DNFB to the ear, the ear thickness is measured using a micrometer. Inflammation (dermatitis) is indicated by a ranked thickening of the ear. Thickness of the treated ear is compared to untreated (contralateral) ear thickness.

EXAMPLE 11: Effect of Antisense Inhibition of TNF-α in a Murine Model for Crohn's Disease

C3H/HeJ, SJL/JK and IL10-/- mice are used in a TNBS (2,4,5,-trinitrobenzene sulfonic acid) induced colitis model for Crohn's disease (Neurath,M.F., *et al.*, *J. Exp. Med.*, **1995**, *182*, 1281-1290). Mice between the ages of 6 weeks and 3 months are used to assess the activity of TNF-α antisense oligonucleotides.

C3H/HeJ, SJL/JK and IL10-/- mice are fasted overnight prior to administration of TNBS. A thin, flexible polyethylene tube is slowly inserted into the colon of the mice so that the tip rests approximately 4 cm proximal to the anus. 0.5 mg of the TNBS in 50% ethanol is slowly injected from the catheter fitted onto a 1 ml syringe. Animals are held inverted in a vertical position for approximately 30 seconds.

Antisense oligonucleotides targeted to TNF-α or PKC-δ which inhibit TNF-α expression are administered either at the first sign of symptoms or simultaneously with induction of disease. Animals, in most cases, are dosed every day. Administration is by i.v., i.p., s.q., minipumps or intracolonic injection. Experimental tissues are collected at the end of the treatment regimen for histochemical evaluation.

EXAMPLE 12: Effect of Antisense Inhibition of TNF-α in a Murine Model for Multiple Sclerosis

Experimental autoimmune encephalomyelitis (EAE) is a commonly accepted murine model for multiple sclerosis (Myers,K.J., *et al.*, *J. Neuroimmunol.*, **1992**, *41*, 1-8). SJL/H, PL/J, (SJLxPL/J)F1, (SJLxBalb/c)F1 and Balb/c female mice between the ages of 6 and 12 weeks are used to test the activity of TNF-α or PKC-δ antisense oligonucleotides.

The mice are immunized in the two rear foot pads and base of the tail with an emulsion consisting of encephalitogenic protein or peptide (according to Myers,K.J., *et al.*, *J. of Immunol.*, **1993**, *151*, 2252-2260) in Complete Freund's Adjuvant supplemented with heat killed Mycobacterium tuberculosis. Two days later, the mice receive an intravenous injection of 500 ng Bordatella pertussis toxin and additional adjuvant.

Alternatively, the disease may also be induced by the adoptive transfer of T-cells. T-cells are obtained from the draining of the lymph nodes of mice immunized with encephalitogenic protein or peptide in CFA. The T cells are grown in tissue culture for several days and then injected intravenously into naive syngeneic recipients.

Mice are monitored and scored daily on a 0-5 scale for signals of the disease, including loss of tail muscle tone, wobbly gait, and various degrees of paralysis.

EXAMPLE 13: Effect of Antisense Inhibition of TNF-α in a Murine Model for Pancreatitis

Swiss Webster, C57BL/56, C57BL/6 lpr and gld male mice are used in an experimental pancreatitis model (Niederau,C., *et al.*, *Gastroenterology*, **1985**, *88*, 1192-1204). Mice between the ages of 4 and 10 weeks are used to assess the activity of TNF-α antisense oligonucleotides.

Caerulin (5-200 μg/kg) is administered i.p. every hour for one to six hours. At varying time intervals, the mice are given i.p. injection of avertin and bled by cardiac puncture. The pancreas and spleen are evaluated for histopathology and increased levels of IL-1β, IL-6, and TNF-α. The blood is analyzed for increased levels of serum amylase and lipase. TNF-α or PKC-δ antisense oligonucleotides are administered by intraperitoneal injection at 4 hours pre-caerulin injections.

EXAMPLE 14: Effect of Antisense Inhibition of TNF-α in a Murine Model for Hepatitis

Concanavalin A-induced hepatitis is used as a murine model for hepatitis (Mizuhara,H., *et al.*, *J. Exp. Med.*, **1994**, *179*, 1529-1537). It has been shown that this type of liver injury is mediated by Fas (Seino,K., *et al.*, *Gastroenterology* **1997**, 113, 1315-1322). Certain types of viral hepatitis, including Hepatitis C, are also mediated by Fas (*J. Gastroenterology and Hepatology*, **1997**, *12*, S223-S226). Female Balb/c and C57BL/6 mice between the ages of 6 weeks and 3 months are used to assess the activity of TNF-α antisense oligonucleotides.

Mice are intravenenously injected with oligonucleotide. The pretreated mice are then intravenously injected with 0.3 mg concanavalin A (Con A) to induce liver injury. Within 24 hours following Con A injection, the livers are removed from the animals and

analyzed for cell death (apoptosis) by *in vitro* methods. In some experiments, blood is collected from the retro-orbital vein.

EXAMPLE 15: Effect of Antisense Inhibition of TNF-α on Survival in Murine Heterotopic Heart Transplant Model

To determine the therapeutic effects of TNF-α or PKC-δ antisense oligonucleotides in preventing allograft rejection, murine oligonucleotides are tested for activity in a murine vascularized heterotopic heart transplant model. Hearts from Balb/c mice are transplanted into the abdominal cavity of C3H mice as primary vascularized grafts essentially as described by Isobe et al., *Circulation* **1991**, 84, 1246-1255. Oligonucleotide is administered by continuous intravenous administration via a 7-day Alzet pump. The mean survival time for untreated mice is usually approximately 9-10 days. Treatment of the mice for 7 days with TNF-α antisense oligonucleotides is expected to increase the mean survival time.